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# SENSITIZING BACTERIAL SPORES TO HEAT BY EXPOSING THEM TO ULTRAVIOLET LIGHT

HAROLD R. CURRAN AND FRED R. EVANS

*Division Dairy Research Laboratories, Bureau of Dairy Industry, U. S. Department of Agriculture*

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Among the many physico-chemical changes produced in proteins by ultraviolet radiation, the changes that affect stability to heat are of particular interest to the biologist. With those rare exceptions noted by Bovie and Woolpert (1924) and Clark (1925) the heat-coagulation temperature of probably all proteins is lowered by the absorption of ultraviolet light. In a study of twelve purified proteins Stedman and Mendel (1926) found that ultraviolet light from a quartz-mercury lamp reduced the temperature of coagulation from 8.5° to 41°C.

Since the killing of living organisms by heat is accompanied by coagulation of the protoplasm it is reasonable to suppose that their heat-resistance would be decreased by ultraviolet radiation. Despite the interesting implications involved, only Bovie and Klein (1919) and Bovie and Daland (1923) have studied the heat-sensitization phenomenon with living microörganisms. Working with Paramecia they observed that sublethal exposures to ultraviolet light, transmitted by fluorite, so affected the organisms that they subsequently died at temperatures which were optimal for the untreated organisms. These striking effects were tacitly assumed to be caused by those rays of very short wave length (Schumann) which are transmitted only by fluorite. While this assumption was undoubtedly correct, these experiments might very naturally lead to the belief that the Schumann rays alone were possessed of heat-sensitizing power. This circumstance, together with certain technical difficulties involved in the generation and transmission of Schumann rays, is perhaps

chiefly responsible for the absence of additional work on this subject.

Consideration of these facts and of the potentialities inherent in the heat-sensitizing principle led us to study the action of ultraviolet rays upon the heat-resistance of bacterial spores. The report which follows presents the results obtained in this investigation.

## EXPERIMENTAL

### *Cultures and Methods*

Three cultures (*Bacillus cohaerens*, *Bacillus albolactis* and CC) were used. The first two were obtained from the American Type Culture Collection. CC, an aerobic spore-former belonging to the *Bacillus mesentericus* group, was isolated in our laboratory from evaporated milk. The cultivation and preparation of the test suspensions have been detailed in a previous paper, by Curran and Evans (1937). The stock and test suspensions when not in use were held in the ice chest.

Two sources of ultraviolet light were used. The first was a cold-mercury-in-quartz lamp of the orificial type. Tests by the National Bureau of Standards upon the spectral energy distribution of this lamp have indicated that at least 95 per cent of the total radiation of all wave lengths less than and including the line at 3,130 Å. is contained in the emission line of mercury vapor at 2,537 Å. Four milliliter quantities of the spore suspension at 30°C. were irradiated in a small quartz balloon flask (18 ml. capacity), placed $2\frac{3}{4}$ inches from the burner. The flask was equipped with a quartz stirrer which provided rapid and uniform agitation. After the exposure to ultraviolet light, equal portions of the spore-suspension were mixed on plates with 15 ml. of agar of the desired composition. Colony-counts were made after 48 hours of incubation at the optimum temperature of the organism. A magnifying glass was used in counting and the figures represent the averages of triplicate plates. From time to time the plates were returned to the incubator, and re-examined 5 days later to check the possibility of slowly developing colonies. The intensity of the cold-quartz radiation was

measured by exposing a sensitive thermopile at a fixed distance from the burner and noting the galvanometer deflections. These values were then converted into absolute energy units by reference to a standard of radiation.

The second source of ultraviolet light was a hydrogen-discharge tube (15 by $1\frac{1}{8}$ inches) fitted with ring electrodes of aluminum. In one end of the tube was sealed a fluorite disc (2 mm. thick) and in the other end a quartz disc of the same thickness. The discs were equi-distant from the electrodes. The tube was excited by a 450-watt luminous tube transformer operating on a 110-volt alternating current of 60 cycles. The current passing through the discharge tube was 33 milliamperes and the drop of potential across the tube was 1,570 volts. The intensity of the radiations transmitted by the tube was not measured. Exposures to this light source were made as follows. A small platinum loopful of a heavy aqueous suspension of the spores was spread uniformly over a 15 x 5 mm. area of a sterile coverslip until dry. The dried film was then applied directly to the window of the discharge tube and exposed. The temperature of the discharge tube windows did not rise perceptibly during the exposures recorded. The spores were recovered in 5 ml. of sterile distilled water; the process of loosening the film from the glass was facilitated by the use of a small, sterile, rubber-tipped glass rod. The suspensions were then thoroughly agitated and plated. The count so obtained served thereafter as a guide for the dilution required to equalize the number of viable cells in the several samples.

Aliquot portions of the standardized samples were heated at 98°C. for 4 minutes and plated.

The medium used was standard nutrient agar of the following composition: 0.5 per cent peptone (Difco), 0.3 per cent beef extract (Liebig's), 0.5 per cent sodium chloride, 1.5 per cent agar (granulated). This basic medium was enriched with either blood or glucose, depending on the nature of the treatment and the enrichment requirements of the organism (See Curran and Evans, 1937).

When bacteria are exposed to both heat and ultraviolet radia-

tion, of constant intensity for the same period of time, the order in which the two treatments are applied should not appreciably affect the number of survivors, if sensitization to neither heat nor light occurs, or if sensitization to both heat and light occurs to the same extent. However, consistent differences in the number of survivors under these conditions do occur and must be attributed to differences in the sensitizing power of the initial

TABLE 1

*The effect of heat (98°C.) and ultraviolet light (21 Ergs/mm²/sec) on viability of bacterial spores, according to the sequence of treatment and time of exposure*

| KIND OF SPORES, AND TIME EXPOSED TO HEAT AND LIGHT | VIABLE SPORES SURVIVING PER MILLILITER WHEN THE SEQUENCE OF TREATMENT WAS: | | SURVIVOR RATIO (H/L : L/H) |
|---|---|---|---|
| | Heat/Light | Light/Heat | |
| *B. cohaerens:** | | | |
| 1½ minutes | 900,000 | 843,000 | 1.0:1.0 |
| 3 minutes | 205,000 | 48,000 | 4.2:1.0 |
| 5 minutes | 3,460 | 420 | 8.2:1.0 |
| 7 minutes | 32 | 13 | 2.4:1.0 |
| CC:† | | | |
| 2 minutes | 400,000 | 346,000 | 1.0:1.0 |
| 3 minutes | 308,000 | 138,000 | 2.2:1.0 |
| 6 minutes | 23,400 | 4,900 | 4.7:1.0 |
| 9 minutes | 392 | 270 | 1.0:1.0 |
| *B. albolactis:*‡ | | | |
| 1 minute | 65,000 | 64,000 | 1.0:1.0 |
| 2 minutes | 2,920 | 1,470 | 1.9:1.0 |
| 3 minutes | 556 | 130 | 4.2:1.0 |
| 3½ minutes | 186 | 30 | 6.2:1.0 |

* Untreated control contained 1,900,000 viable spores per ml.
† Untreated control contained 2,100,000 viable spores per ml.
‡ Untreated control contained 350,000 viable spores per ml.

lethal agent. The results presented in Table I were obtained when spores uniformly dispersed in distilled water were exposed to radiation from a quartz-mercury lamp and heat of constant intensity in the order, and for the periods, indicated. Changes in the reaction of the spore suspension during irradiation did not exceed 0.1 pH; hence this factor may be disregarded in the interpretation of results. These data clearly show that the spore-

killing action of any given combination of heat and ultraviolet light is materially influenced by the order of their application.

When the periods of exposure were short and the mortality relatively low, the order of treatment had but little influence upon the number of spores which survived; a slight but distinctly greater mortality resulting when heat succeeded irradiation. With lengthening of the exposure periods and consequent rereduction in number of survivors, the order of treatment became of increasing significance. Comparative differences are indicated by changes in the survivor ratio. Light preceding heat was always more destructive than heat preceding light. As may be noted in table 1, the survivor ratio increased with length of the exposures until maximum sensitization was attained. With some species, this was followed by a reduction in the survivor ratio; with one species such an effect was not observed.

The spores of the three species reacted in essentially the same way to the combined action of irradiation and heat. The sensitizing effect was greatest with the spores of *B. cohaerens* in which the survivor ratio at its maximum was more than 8:1. The increased mortality attributable to sensitization, manifestly represents only a very small part of the total population but it is significant that the spores most easily sensitized are those of high resistance.

The change produced in spores by exposure to ultraviolet light is apparently irreversible since their tolerance to heat was found to be independent of the time which elapsed between the two treatments.

The heat-sensitizing action of ultraviolet light is again brought out in table 2. Under the conditions of the experiment, irradiation for 2 minutes was about equal to heat for 5 minutes in sporocidal action. By making a suitable dilution of the untreated suspension, a control suspension of spores could be obtained in which the number of viable spores was comparable with the number surviving a 5 minute heating period or 2 minutes' irradiation. To the control suspension was added a sufficient amount of heat-killed spores to make the total number of living and dead spores essentially the same in the three samples.

Under these conditions, when an irradiated suspension was given a second exposure to light only 100,000 spores survived. When the suspension was first heated and then irradiated, over six times as many spores survived and this number was about equal to the number that survived a single exposure to ultraviolet light when the suspension was neither heated nor irradiated previously. The spores previously heated were, therefore, no more sensitive to irradiation than spores not previously heated or irradiated. When heated spores were given a second heating the mortality was somewhat greater than for the light-heat combination, but the control spores in this instance were consider-

TABLE 2

*The effect of ultraviolet light (incident energy 22.2 Ergs/mm²/sec.) and of heat (98°C.) upon spores of B. cohaerens*

| VIABLE SPORES PER MILLILITER IN UNTREATED SUSPENSION | VIABLE SPORES PER MILLILITER WHEN THE SEQUENCES AND TIMES OF TREATMENT WERE | | | | | |
|---|---|---|---|---|---|---|
| | First treatment | Time | Count | Second treatment | Time | Count |
| | | min. | | | min. | |
| 2,700,000 | Light | 2 | 770,000 | Light | 2 | 100,000 |
| | | | | Heat | 5 | 230,000 |
| | Heat | 5 | 860,000 | Light | 2 | 650,000 |
| | | | | Heat | 5 | 180,000 |
| | Dilution | | 840,000 | Light | 2 | 540,000 |
| | | | | Heat | 5 | 450,000 |

ably more resistant to heat than were the spores that had been irradiated.

It is apparent that spores which survive lethal heat or lethal ultraviolet light are sensitized to that particular form of energy. Although the results are somewhat variable the general relationships were consistently obtained under conditions of adequate irradiation. Thus, where heat and ultraviolet light of about the same sporocidal power are concerned, sensitization to light is best produced by light, and sensitization to heat is best produced by heat. When combinations are involved, light sensitizes to heat less than heat does to heat. Since heated spores are no more sensitive to irradiation than spores that have not been

first heated or irradiated, heat may be regarded as having little or no light-sensitizing action. In the foregoing experiments the spores were exposed in a fused quartz vessel, hence the effects observed were produced by rays of wave lengths longer than 2,000 Å.

Since the effects described by Bovie *et al.* were believed to be caused by ultraviolet rays of very short wave length it seemed of particular interest to compare the heat-sensitizing action of long and very short ultraviolet rays. In order to accomplish this, the spores were dried on sterile coverslips and during exposure pressed tightly against the fluorite and quartz windows of a hydrogen discharge tube. After exposure, the spores were collected and treated as previously described. Ozone effects may be considered negligible owing to the exclusion of air between the discharge tube windows and the spores.

Examination of table 3 shows that spores not previously exposed to ultraviolet light were clearly much more resistant to heat than those previously exposed to light passing through fluorite or quartz. The objection may be made that spores which survive a destructive influence might reasonably be expected to be so weakened that they would die more rapidly when exposed to another destructive influence. However, reasonable as this supposition may seem, it is not universally true. As pointed out in connection with the study of the light-heat sequence in table 2, spores which survived heat were no more sensitive to ultraviolet light than those not previously exposed to heat. We believe, therefore, that irradiated spores are more susceptible to heat because of a specific change in the condition of the cells produced by the ultraviolet rays. The heat-sensitizing effects of the rays passing through fluorite and quartz as shown in the table are not directly comparable because of differences in transparency of the two substances. With equal exposures somewhat more energy would be emitted at the fluorite window.

In an effort to obtain results which would serve as a more satisfactory basis for comparison, the exposures through quartz were lengthened by 5 second increments up to 30 seconds. Ex-

amination of the results obtained shows that the light transmitted by quartz was much less effective in sensitizing the spores to heat even with much longer exposures. These effects are best observed in the percentage reduction values. Only when the exposure through quartz was three times that through fluorite were the heat-sensitizing effects similar. From this it is evident that ultraviolet rays of very short wave length have much greater heat-sensitizing action upon spores than those transmitted by quartz. The sample of fluorite used was transparent to 1,250 Å.

TABLE 3

*The influence of ultraviolet light transmitted by quartz and fluorite upon the heat-resistance of B. cohaerens spores*

| ULTRAVIOLET TREATMENT OF B. COHAERENS IN ORIGINAL SAMPLES | VIABLE SPORES PER MILLILITER | | PERCENTAGE REDUCTION BY HEATING |
|---|---|---|---|
| | In original samples after irradiation | In irradiated samples after heating for 4 minutes at 98°C. | |
| | | | *per cent* |
| No treatment (control) | 368 | 181 | 50.8 |
| Fluorite, for 10 seconds | 445 | 40 | 91.0 |
| Quartz: | | | |
| For 10 seconds | 430 | 153 | 64.4 |
| For 15 seconds | 389 | 94 | 75.8 |
| For 20 seconds | 421 | 77 | 81.7 |
| For 30 seconds | 407 | 40 | 90.1 |
| Fluorite, plus quartz for 10 seconds | 380 | 151 | 60.2 |
| Quartz, plus quartz for 10 seconds | 385 | 151 | 52.3 |
| Fluorite, for 10 seconds at 5 mm | 440 | 211 | 52.0 |
| Quartz, for 10 seconds at 5 mm | 430 | 201 | 53.2 |

Crystal quartz of the thickness used is transparent to 1,600 Å., according to Lyman (1914), hence the effects observed are to be attributed to the Schumann rays through a spectral range of 350 Å.

In this study some exposures were made in which a second quartz disc 2 mm. thick was interposed between the spores and the discharge tube windows. So treated, the heat-sensitizing effects in the two combinations were almost identical and both somewhat less than those obtained with light transmitted by the

single quartz disc. The second quartz disc absorbs completely the shorter rays passing through fluorite and the exclusion of these rays reduces the heat susceptibility of the spores to about 60 per cent.

In order to confirm the belief that the heat-sensitizing action of the rays transmitted by fluorite was caused by the very short rays in the Schumann region the spores were exposed 5 mm. from the window. An air column of this thickness will absorb practically all the Schumann rays as pointed out by Pflüger (1904). Increasing the path of light does, of course, reduce the light intensity, but the decreased sensitization resulting therefrom is obviously much greater than can be explained by the inverse-square law. In calculations based on the inverse-square law the reduction in intensity could not exceed 12 per cent, and inasmuch as this is not a point source, the reduction in intensity is less (of the order of 4 to 6 per cent), whereas the observed reduction in heat susceptibility is from 91 per cent to 52 per cent.

The data recorded in this table represent the averages of 10 separate experiments in which the deviation from the average was approximately $\pm$ 4.5.

## DISCUSSION

In this study, sensitization to heat was observed only under conditions which were sporocidal for a considerable number of cells. Sublethal exposures did not measurably decrease the heat-resistance of spores so treated. It is apparent that there is a minimum level of radiant energy below which sensitization does not occur. Where the weaker cells are concerned this threshold value may exceed their ultraviolet light tolerance; this we believe to be the most plausible explanation for the low sensitization which accompanies low mortality.

The exact mechanism by which ultraviolet light renders bacteria more susceptible to heat is a matter for conjecture. A satisfactory explanation may be found in the reactions known to occur in simple proteins under the influence of ultraviolet radiation.

Clark (1935) has demonstrated that the coagulation of iso-

electric egg albumin solutions by ultraviolet radiation involves three distinct processes; first, a physical process, independent of temperature and largely independent of hydrogen-ion concentration; secondly, a chemical reaction between the light-denatured molecule and water, which is similar in some respects to denaturation by heat, and finally flocculation of the previously-altered molecules. The significant fact observed by Clark was that heat-denaturation, the precursor of flocculation, occurs at a lower temperature after light-denaturation; in the latter state, therefore, the protein must be in a chemically active condition.

By analogous reasoning, bacterial protoplasm is converted by the absorption of ultraviolet energy into a more reactive and therefore less resistant state, in consequence of which the subsequent processes of heat-denaturation and flocculation occur at lower energy levels.

The permanent nature of light-denaturation is in accord with our observation previously described in which heat sensitization effects were found to be independent of the time elapsing between irradiation and heat treatment. The irreversibility of the reaction with Paramecia appears to be open to question since Bovie and Klein (1918) observed complete recovery from the effects of Schumann rays when sufficient time elapsed before heat was applied.

## SUMMARY

Many spores which survive lethal heat or ultraviolet radiation are thereby sensitized to that particular influence and in consequence are more easily killed by further applications of the same treatment than are untreated or control spores.

Many spores which survive lethal ultraviolet radiation are thereby sensitized to heat. The number of spores sensitized to heat by ultraviolet radiation is less than the number sensitized to light by ultraviolet radiation; similarly the number of spores sensitized to heat by ultraviolet radiation is less than the number sensitized to heat by heat.

Apparently not all spores are susceptible to sensitization by

heat; those moderately and highly resistant to light and heat are most affected.

When spores are exposed to the combined action of heat and ultraviolet radiation the order of treatment materially affects the number which will survive. Under conditions of adequate irradiation, the light-heat sequence is always the more destructive.

Heat has no appreciable light-sensitizing action upon spores.

Ultraviolet rays of wave lengths longer than 2,000 Å., under suitable conditions of exposure, sensitize spores to heat. Ultraviolet rays in that portion of the Schumann region between 1,250 and 1,600 Å. are more effective in sensitizing spores to heat than those transmitted by quartz.

It is a pleasure to acknowledge our indebtedness to Dr. W. W. Coblentz of the National Bureau of Standards for his counsel in regard to air-space absorption and analysis of these data, and to Dr. G. R. Greenbank of these laboratories for assistance in radiometric measurements.

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# COMPARATIVE METABOLISM OF THE R AND S VARIANTS OF LACTOBACILLUS PLANTARUM (ORLA-JENSEN)

RALPH L. TRACY

*Department of Bacteriology, School of Medicine, University of Southern California, Los Angeles, California*

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The extreme variability in fermentation reactions of lactobacilli is well known. Weiss and Rettger (1934), Curran, Rogers and Whittier (1933), and Howitt (1930) have ably reviewed this subject.

The alterations in fermentative reactions have seldom however been clearly correlated with colonial dissociation. Roos (1927) isolated from one strain of *Lactobacillus acidophilus* three distinct fermentative and colonial types. Hadley, Bunting and Delves (1930) found that R variants obtained from S strains of oral aciduric bacteria fermented glucose readily but differed slightly in their reactions to the S type on other sugars. R variants, designated as group III, fermented glucose and showed depressed activity with other sugars. Curran, Rogers and Whittier (1933) separated aciduric strains by correlating colony form, effect of temperature on growth, type of lactic acid produced and fermentation reaction. Kopeloff and Kopeloff (1937) observed that R variants of *L. acidophilus* produced inactive lactic acid while the S type produced *d* lactic acid. R variants of *Lactobacillus bulgaricus* developed both *d* lactic and inactive lactive acid.

The present study demonstrates a correlation of colony type and fermentative reaction of *Lactobacillus plantarum*. A marked depression of carbohydrate activity along with a definite enhancement of non-saccharolytic properties is noted in the dissociation of S to R.

## ORIGIN OF CULTURE

The lactobacillus used in this study was originally isolated as the S type from olive brine (Tracy, 1934). During the several

years this organism has been serially cultivated in glucose broth, it has shown enhancement of fermentative capacities. Originally it failed to ferment lactose, raffinose, arabinose and dextrin. Now, however, these carbohydrates are readily fermented.

Following the classification of Pederson (1936), this strain has been designated *Lactobacillus plantarum*. The properties exhibited by it, even to the limits of salt tolerance, closely approximate the characters presented by Dr. Pederson for the species, *L. plantarum* (Orla-Jensen).

## DISSOCIATION

The most reliable method developed for the dissociation of S to R was the usual rapid serial cultivation of the S type in 1 per cent glucose broth followed by plating (streak method) on glucose agar containing 4 per cent NaCl. Dissociation in this manner appeared to be abrupt, as intermediate colonial types were seldom encountered. LiCl broth or agar failed to induce dissociation from S to R. Ageing in gelatin, glucose agar, glucose broth, serial transfer in $FeCl_3$ (0.1 per cent) broth or agar irregularly induced S to R variation.

R to S dissociation has not been accomplished. The dissociative path of this species of lactobacillus was directly opposite to that of *L. acidophilus* as shown by Upton and Kopeloff (1932) and Raney and Kopeloff (1934) who consistently failed to induce S to R variation but readily produced R to S transformation.

Stock strains of the R type were very stable, and have been subcultured continuously in 1 per cent glucose broth, or glucose broth containing 4 per cent NaCl. Stock strains of the S type were stable when subcultured once or twice weekly in glucose broth. Occasionally "accidental" ageing (2 weeks) induced dissociation, especially in mannitol and fructose broth.

## FERMENTATION OF CARBOHYDRATES BY R AND S VARIANTS

### *Growth in glucose broth*

Both R and S variants grew luxuriantly in 1 per cent glucose broth. The lag period of growth was shorter for the S type, and

the maximum number of cells produced was greater by both total and viable counts. In general, the total count for the S type at 37°C. was $4 \times 10^9$ cells per cubic centimeter and for the R type it was $5 \times 10^8$ cells per cubic centimeter although not uncommonly $10^9$ cells per cubic centimeter were produced.

Both R and S types settled completely after maximum growth. The S type of sediment was loose while the R type was stringy and mucus-like. Growth was similar in glucose broth containing 4 per cent NaCl, except that longer lag periods were noted, and less cells per cubic centimeter were present at maximum growth.

Microscopically, cells of the S type were evenly dispersed throughout the field, while the R type of cells, which invariably occurred in small, tight clusters or packets of 5 or 10 cells each, were unevenly distributed. This character of the R type interfered with obtaining reliable bacterial counts.

*Acid production in glucose broth*

Although the S variant readily produced acid when grown in glucose broth, the R variant failed to produce any titrable acid. If the R variant was incubated for a prolonged period of time, however, titrable acid would irregularly be detected in small quantities.

In table 1, data are presented comparing the acid production of R and S variants grown in 1 per cent glucose broth.[1] The data show that titrable acid is not produced by the R variant even after maximum growth is reached, although the S variant produces titrable acid as early as 12 hours after initial inoculation.

In this test, as in following ones, titrations for total acid were made on 1 cc. samples of glucose broth. The burette containing base was 10 cc. in capacity, graduated to 0.05 cc. volumes. The pH determinations were made with a Leeds and Northrup quinhydrone apparatus. Bacterial counts were made by serially diluting 1 cc. samples of culture in glucose broth, then plating on glucose agar as usual. Both the plates and dilution tubes

[1] These protocols are individual tests on the R and S variants among a large number of similar experiments made for the purpose of determining maximum zymase activity. All tests have the trend shown in table 1.

were incubated at 37°C. for 4 to 5 days before counting. Close agreement between positive tubes and plate counts usually was obtained.

An R variant, subcultured in 1 per cent glucose broth containing either 1 or 4 per cent of NaCl for 34 and 64 subcultures respectively, failed to produce titrable acid when the incubation period at 37°C. between subcultures was 50 hours or less. In table 2, data are presented showing the acid production in these strains when the incubation period between subcultures exceeded 50 hours.

TABLE 1

*Production of acid by R and S variants in 1 per cent glucose broth*

| TIME OF FERMENTATION AT 37°C. | NUMBERS OF VIABLE CELLS PER CC. | | N/10 NaOH TO NEUTRALIZE 1 CC. SAMPLE OF BROTH PHENOLPHTHALEIN INDICATOR | | pH OF BROTH | | TOTAL ACID IN TERMS OF LACTIC ACID PER CC. OF BROTH | | ACID PRODUCED PER CC. IN TERMS OF LACTIC ACID | |
|---|---|---|---|---|---|---|---|---|---|---|
| | R | S | R | S | R | S | R | S | R | S |
| *hours* | | | *cc.* | *cc.* | | | | | *mgm.* | *mgm.* |
| 0 | $10^5$ | $10^5$ | 0.22 | 0.20 | 6.1 | 6.1 | 1.98 | 1.80 | 0.00 | 0.00 |
| 4 | | $10^6$ | 0.21 | 0.20 | 6.3 | | 1.89 | 1.80 | 0.00 | 0.00 |
| 8 | $10^6$ | $10^7$ | 0.21 | 0.20 | | 6.1 | 1.89 | 1.80 | 0.00 | 0.00 |
| 12 | $10^7$ | $10^8$ | 0.20 | 0.23 | | 5.8 | 1.80 | 2.07 | 0.00 | 0.27 |
| 16 | $10^8$ | $10^8$ | 0.20 | 0.24 | 6.3 | | 1.80 | 2.16 | 0.00 | 0.36 |
| 20 | $10^8$ | $10^9$ | 0.24 | 0.30 | | 5.2 | 2.16 | 2.70 | 0.18 | 0.90 |
| 22 | $10^9$ | $10^9$ | 0.21 | 0.32 | | 4.9 | 1.89 | 2.88 | 0.00 | 1.08 |
| 24 | $10^9$ | $10^9$ | 0.21 | 0.33 | 6.1 | 4.9 | 1.89 | 3.07 | 0.01 | 1.27 |
| 30 | $10^9$ | $10^9$ | 0.20 | 0.36 | 6.5 | 4.7 | 1.80 | 3.24 | 0.00 | 1.44 |

The values show that acid may be produced by the R variants when prolonged incubation periods in glucose broth are maintained. Nevertheless, the quantity of acid produced in this period is equivalent only to that formed by the S variant in 12 to 20 hours. Only R variants have been isolated from these acid-forming R cultures.

In table 3, data are presented to show that the S variant readily uses glucose simultaneously with acid production, while the R variant fails to utilize glucose and is unable to form acid. The Schaffer-Hartmann method was used to determine the presence

TABLE 2

*Acid production by R variant continuously subcultured in glucose broth*

| TIME OF INCUBATION AT 37°C. | DIFFERENCE IN INITIAL AND FINAL TITRATIONS OF N/10 NaOH | | | | TOTAL ACID PRODUCED IN TERMS OF LACTIC ACID PER CC. | |
|---|---|---|---|---|---|---|
| | 1% glucose broth | | | | 1% glucose broth | |
| | 1% NaCl | | 4% NaCl | | 1% NaCl | 4% NaCl |
| | R | Control | R | Control | R | R |
| *hours* | *cc.* | *cc.* | *cc.* | *cc.* | *mgm.* | *mgm.* |
| 53 | 0.01 | 0.02 | 0.00 | −0.02* | 0.00 | 0.00 |
| 67 | 0.01 | −0.04 | 0.00 | −0.03 | 0.00 | 0.00 |
| 76 | 0.03 | −0.03 | 0.00 | 0.01 | | 0.00 |
| 94 | −0.02 | 0.04 | 0.02 | −0.01 | 0.00 | |
| 99 | 0.01 | 0.01 | 0.00 | −0.03 | 0.00 | 0.00 |
| *days* | | | | | | |
| 8 | −0.03 | 0.04 | 0.02 | 0.04 | 0.00 | 0.00 |
| 8.5 | 0.04 | 0.01 | 0.01 | −0.03 | 0.27 | |
| 9 | −0.03 | 0.03 | 0.00 | 0.01 | 0.00 | 0.00 |
| 10 | 0.08 | 0.02 | 0.13 | 0.04 | 0.54 | 0.81 |
| 11.5 | 0.06 | 0.06 | 0.09 | 0.04 | 0.00 | 0.45 |
| 26 | 0.24 | 0.20 | 0.18 | 0.13 | 0.36 | 0.45 |

* Final titration more alkaline than initial titration by −0.02 cc. Average experimental error in titration ±0.05 cc.

TABLE 3

*Utilization of glucose by R and S variants grown in 1 per cent glucose broth*

| | R VARIANT | | | | | | | S VARIANT | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Time of incubation an 37°C. | | | | | | | | | | | | | |
| | 0 hours | | | 24 hours | | | 8 days | 0 hours | | | 24 hours | | | 8 days |
| | Test 1 | Test 2 | Test 3 | Test 1 | Test 2 | Test 3 | Test 3 | Test 4 | Test 5 | Test 6 | Test 4 | Test 5 | Test 6 | Test 6 |
| Total acid in terms of mgm. of lactic acid per cc. | 1.98 | 1.98 | 2.3 | 1.98 | 1.8 | 2.3 | 3.0 | 1.80 | 1.80 | 1.80 | 3.87 | 3.0 | 4.1 | 8.2 |
| Reducing substances in terms of mgm. of glucose per cc. | 10.92 | 10.92 | 12.60 | 10.92 | 10.87 | 12.90 | 13.10 | 10.00 | 10.50 | 12.80 | 8.57 | 9.38 | 10.10 | 5.2 |
| Mgm. of glucose utilized per cc. | | | | 0.00 | 0.05 | 0.00 | 0.00 | | | | 1.43 | 1.12 | 2.70 | 7.60 |
| Mgm. of acid formed per cc. in terms of mgm. of lactic acid | | | | 0.00 | 0.00 | 0.00 | 0.07 | | | | 2.07 | 1.20 | 2.3 | 6.4 |

of reducing substances in the culture media. In most tests, proteins were not precipitated from the culture media before testing for reducing substances.

In table 4, data are given which show that lactic acid is formed by the S variant but is not formed by the R variant. The method of Friedmann, Cotonio, and Schaffer, as modified by Wendel (1935) was used to test for the presence of lactic acid.[2]

### *Acid production in different carbohydrates*

When the R and S variants were grown in broth containing carbohydrates other than glucose, titrable acid was formed by the S type but not by the R variant. Data are given in table 5.

TABLE 4

*Production of lactic acid by R and S variants in 1 per cent glucose broth*

| | TIME OF INCUBATION AT 37°C. | | | | |
|---|---|---|---|---|---|
| | 24 hours | | Broth control | 48 hours | |
| | R variant | S variant | | R variant | S variant |
| Total titrable acid produced in terms of mgm. of lactic acid per cc. | 0.09 | 3.78 | 0.00 | 0.09 | 4.86 |
| Mgm. of lactic acid per cc. | | 5.9 | 2.7 | 2.3 | 6.4 |
| | | 6.0 | 3.0 | 2.5 | 6.8 |
| Mgm. of lactic acid produced per cc. | | 3.10 | | 0.00 | 3.75 |

These results indicate that the R variant is generally devoid of active carbohydrases. Growth of the S variant in each of these media was luxuriant and produced approximately equal turbidity in the different compounds; the R variant, however, grew sparingly in each medium. The S variant had been continuously subcultured in glucose broth since initial isolation. The glucose R variant had been subcultured serially in glucose broth for 20 transplants, while the sugar-free R variant had been carried in sugar-free broth for 49 transfers. Sugar-free broth

[2] Mr. Charles Lombard, chemist, Los Angeles County Hospital, directed and generously aided in these lactic acid determinations.

was prepared by mixing 1 per cent each of beef extract (Difco), NaCl, and sugar-free peptone in distilled water. Sugar-free peptone was prepared by the usual method of incubating, at 37°C., heavy suspensions of *Escherichia coli* in 10 per cent peptone solutions for 48 to 72 hours under vaseline seal.

### FERMENTATION OF CARBOHYDRATES BY "RESTING" CELL SUSPENSIONS

In order to exclude the possibility that insufficient concentration of cells in the R type of culture prevented the development of acid, the production of acid by "resting" cell suspensions was

TABLE 5

*Production of titrable acid by R and S variants grown in various carbohydrate broths**

| VARIANT GROWN IN SPECIFIC CARBOHYDRATE | TIME OF INCUBATION AT 37°C. | ARABINOSE | FRUCTOSE | MANNOSE | GALACTOSE | SUCROSE | LACTOSE | RAFFINOSE | GLYCEROL† |
|---|---|---|---|---|---|---|---|---|---|
| | | Total acid in terms N/10 NaOH in cc. | | | | | | | |
| | hours | | | | | | | | |
| Glucose S | 72 | 0.15 | 0.61 | 0.48 | 0.59 | 0.27 | 0.12 | 0.18 | 0.11 |
| Glucose R | 72 | 0.00 | −0.05 | 0.00 | 0.01 | 0.01 | −0.05 | 0.03 | −0.04‡ |
| Sugar-free R | 72 | 0.00 | 0.02 | 0.02 | 0.03 | 0.03 | −0.03 | −0.02 | −0.03 |

* Nutrient broth containing 1 per cent of a given carbohydrate. Experimental error in titration = ±0.05 cc.

† Included as the three carbon compound since glycerose was unavailable.

‡ Final titration more alkaline than initial titration by 0.04 cc. N/10 NaOH.

tested. R and S variants were grown in broth containing 1 per cent carbohydrate, centrifuged and "washed" in saline 3 times. Arabinose, glucose, lactose and raffinose were the carbohydrates used except in the case of the R variant which was grown only in glucose broth. These cells were added to solutions of various carbohydrates in 0.01 M phosphate buffer and 0.01 M phosphate buffer plus 1 per cent peptone so that the final concentration was 10 mgm. of cells (wet weight) per cubic centimeter. The initial pH in each tube was 6.2. The results of this test are presented in table 6.

Although the average experimental error in titrating was

somewhat large (±0.07), the values clearly indicate that specific carbohydrases were developed by the S variant for the particular substrate in which it had been grown except in the case of glucose. Interpretation of the data in accordance with Karstrom (1930), suggests that zymase of the S variant is constitutive in character

TABLE 6

*Fermentation of various carbohydrates by R and S variants grown in specific sugars*

| VARIANT GROWN IN SPECIFIC CARBOHYDRATE | FERMENTATION AT 37°C. | TOTAL ACID IN TERMS OF N/10 NaOH | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Phosphate buffer | | | | Phosphate buffer + 1 per cent peptone | | | |
| | | Arabinose | Glucose | Lactose | Raffinose | Arabinose | Glucose | Lactose | Raffinose |
| | *hours* | *cc.* | *cc.* | *cc.* | *cc.* | *cc.* | *cc.* | *cc.* | *cc.* |
| Arabinose S | 2 | 0.10 | 0.10 | 0.00 | 0.00 | 0.24 | 0.24 | 0.04 | 0.00 |
| | 5 | 0.17 | 0.20 | 0.03 | 0.00 | 0.30 | 0.35 | 0.06 | 0.04 |
| Glucose S | 2 | 0.02 | 0.07 | 0.00 | 0.00 | 0.01 | 0.12 | 0.03 | 0.01 |
| | 5 | 0.02 | 0.10 | 0.02 | 0.03 | 0.05 | 0.16 | 0.04 | 0.03 |
| Lactose S | 2 | 0.00 | 0.12 | 0.06 | 0.00 | 0.03 | 0.11 | 0.11 | 0.05 |
| | 5 | 0.00 | 0.16 | 0.30 | 0.03 | 0.02 | 0.26 | 0.21 | 0.07 |
| Raffinose S | 2 | 0.07 | 0.12 | 0.06 | 0.10 | 0.00 | 0.20 | 0.10 | 0.22 |
| | 5 | 0.03 | 0.26 | 0.19 | 0.14 | 0.02 | 0.26 | 0.27 | 0.37 |
| Glucose R | 2 | 0.00 | 0.03 | 0.00 | 0.06 | 0.00 | 0.01 | 0.03 | 0.00 |
| | 6 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.06 | 0.07 | 0.03 |
| Control titration without bacteria | 2 | 0.00 | −0.03 | −0.04 | −0.02 | −0.01 | −0.07 | 0.00 | 0.03 |
| | 5 | 0.00 | 0.00 | 0.00 | −0.03 | −0.04 | −0.05 | −0.01 | 0.03 |
| | 6 | −0.02* | −0.06 | −0.03 | 0.03 | −0.04 | 0.07 | −0.02 | 0.00 |

* Final titration was more alkaline than initial titration by 0.02 cc. N/10 NaOH. Experimental error ±0.07.

while the enzymes attacking arabinose, lactose and raffinose are adaptive. The R variant failed to produce titrable acid in any of the carbohydrates tested. Although the titrable acid was greater in the carbohydrate solutions containing peptone in nearly every case with the S variant, the R form was unaffected by peptone.

### GROWTH OF R AND S VARIANTS IN CARBOHYDRATE-FREE MEDIA

That lactobacilli have a distinct saccharolytic type of metabolism has been well known since Rahe (1914 and 1918) emphasized that aciduric bacteria gave luxuriant growth in unneutralized meat-peptone broth only when suitable carbohydrates were added. Weaver (1932) noted that *L. acidophilus* and *L. bulgaricus* did not grow in sugar-free basic media. However, proteolytic activities of lactobacilli have been noted by Gorini (1922), Kendall and Haner (1924a, b), Kulp and Rettger (1924), Peterson, Pruess and Fred (1928), and Frazier and Rupp (1931).

When the R and S variants were cultured in nutrient broth, the R variant readily grew but the S variant developed only sparingly. Serial cultivation of the S type was possible usually for three transfers in this medium; the R variant, however, could be transferred continuously without difficulty. When these strains were cultured in sugar-free broth, the S form failed to grow but the R variant readily developed. In each of these tests, the inocula were "washed" cell suspensions grown in glucose broth. The initial concentration of cells in the test media approximated $10^7$ cells per cubic centimeter although $10^5$ cells per cubic centimeter were often used for the R type.

In table 7, growth of the variants in different media is summarized. The data show that not only will the R form grow in sugar-free peptone solutions while the S type fails to do so, but also that the R variant can grow in a smaller concentration of peptone than the S form even when glucose is available.

Further behavior of these variants is shown in table 8. The phosphotungstic acid precipitate of an acid alcohol extract of peptone was prepared according to the method of Sahyun, *et al.* (1936). Butyl alcohol fractions of the extract were not prepared. The casein was dissolved in N/10 NaOH, diluted, and neutralized to pH 7 with weak HCl. The data show that the R variant grows in the alcoholic extract of peptone and in casein in the absence of glucose while the S form grows in the extract only when glucose is present. The S form does not grow in the casein

medium even in the presence of glucose. These results suggest that the R variant possesses a greater proteolytic activity than the S type. However, as growth of the R variant in casein was slow and extremely slight, it is possible that only hydrolysis products of casein, formed on dissolution in alkali, acted as nutrients

TABLE 7

*Comparison of growth of R and S variants in sugar-free and sugar-containing media**

| MEDIUM | S VARIANT | | R VARIANT | |
|---|---|---|---|---|
| | Time of incubation at 37°C. | Growth | Time of incubation at 37°C. | Growth |
| | days | | days | |
| Sugar-free broth | 7 | − | 2 | ++++ |
| Sugar-free broth + 1 per cent glucose | 2 | ++++ | 2 | ++++ |
| Sugar-free peptone† in per cent: | | | | |
| 1.0 | 7 | − | 2 | ++++ |
| 0.1 | 7 | − | 7 | +++ |
| 0.075 | 7 | − | 7 | Trace |
| 0.05 | 7 | − | 7 | −; +‡ |
| Sugar-free peptone + 1 per cent glucose in per cent of peptone: | | | | |
| 1.0 | 2 | ++++ | 2 | ++++ |
| 0.1 | 7 | + | 7 | +++ |
| 0.075 | 7 | − | 7 | + |
| 0.05 | 7 | − | 7 | −; + |

* Growth is recorded according to the turbidity developed in the medium. No attempt was made to compare quantitatively the growth of the R and S variants.

† This medium was prepared by diluting sugar-free peptone in a basal salt solution of the composition in per cent as follows: $K_2HPO_4$, 0.17; $KH_2PO_4$, 0.14; $MgSO_4 \cdot 7H_2O$, 0.02; $CaCl_2$, 0.01; NaCl, 0.5.

‡ −; + indicates that in some tests growth occurred although in the majority of tests no growth was noted.

in this medium. This is in accord with the observation that the R variant requires a smaller concentration of peptone for growth than the S variant.

Nevertheless, these tests with carbohydrate-free media indicate that a marked difference in the metabolism of the two variants exists. This difference is especially pronounced when the utiliza-

tion of carbohydrates is considered. The observations indicate that the S variant possesses a saccharolytic metabolism with little or no ability to utilize nitrogenous compounds in the absence of carbohydrates while the R variant possesses a nitrogenous metabolism devoid of saccharolytic activities. Although the latter retains or gains some ability to utilize glucose as is evident after prolonged incubation in glucose broth, this behavior is not significant from the viewpoint of being essential for growth.

TABLE 8

*Growth of R and S variants in alcoholic extract of peptone and in casein solutions*

| MEDIA* | CONCENTRATION OF $N_2$ COMPOUNDS | TIME OF INCUBATION AT 37°C. | GROWTH OF VARIANTS† | |
|---|---|---|---|---|
| | | | R | S |
| | *mgm. per cc.* | *days* | | |
| Extract | 10.0 | 4 | ++++ | − |
| | 1.0 | 4 | ++ | − |
| | 0.1 | 5 | + | − |
| Extract + glucose (1 per cent) | 10.0 | 4 | ++++ | ++++ |
| | 1.0 | 4 | ++ | ++ |
| | 0.1 | 5 | ± | − |
| Casein‡ | 10.0 | 5 | + | − |
| Casein + glucose (1 per cent) | 10.0 | 5 | + | − |
| Casein + glucose (1 per cent) + peptone (1 per cent) | 10.0 | 2 | ++++ | ++++ |

* Prepared by dissolving the alcoholic extract of peptone and the casein in basal salts solution.

† Inocula were cells cultured in glucose broth for 24 hours, and washed once in saline. Initial concentration of cells in test media approximated $10^7$ per cc.

‡ "Labco" purified, vitamin-free casein.

Of interest in this connection are the studies of both Hoogerheide (1937) who obtained from a non-saccharolytic strain of *Clostridium histolyticum*, a saccharolytic variant similar to *Clostridium sporogenes*, and Goodman (1908) who developed a non-acid producing strain of *Corynebacterium diphtheriae* from a normal toxogenic stock culture. It is concluded that by the dissociation of *L. plantarum* from S to R, a saccharolytic bacterium is changed to a non-saccharolytic type possessing enhanced capacities for nitrogenous nutrients.

## SUMMARY AND CONCLUSIONS

1. It was found that rapidly growing cultures of *Lactobacillus plantarum* would dissociate from S to R when plated on glucose agar containing 4 per cent NaCl.

2. The R variant failed to produce titrable acid when grown for 1 to 8 days at 37°C. in 1 per cent glucose broth, although the S variant rapidly produced acid in this medium. The R variant produced detectable quantities of acid in glucose broth, however, after prolonged incubation periods (8 to 26 days).

3. The R variant did not utilize glucose when grown in glucose broth at 37°C. for 8 days, although the S variant utilized approximately 60 per cent of the available glucose in this medium in 8 days.

4. Lactic acid was produced by the S variant but was not formed by the R variant.

5. Although the R variant was grown at 37°C. for 72 hours in broth containing carbohydrates other than glucose, titrable acid was not produced. The S variant readily fermented these carbohydrates.

6. "Resting" cell suspensions of the R variant consisting of approximately 10 mgm. of cells per cubic centimeter failed to form titrable acid in arabinose, glucose, lactose and raffinose. Similar suspensions of the S variant produced acid with these carbohydrates.

7. It was found that the R variant grew in sugar-free peptone solutions or broth. The S variant failed to grow in these media in the absence of carbohydrates.

8. The R variant but not the S type grew in carbohydrate-free solutions of casein or an alcoholic extract of peptone. The S variant grew in the alcoholic extract of peptone when glucose was present but failed to grow in casein medium containing glucose.

9. It is concluded that in the dissociation of *Lactobacillus plantarum* from S to R, a saccharolytic bacterium is changed into a non-saccharolytic type possessing enhanced capacities for nitrogenous nutrients.

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# THE BACTERICIDAL EFFECT OF SULFANILAMIDE UPON BETA HEMOLYTIC STREPTOCOCCI IN VITRO

HAROLD J. WHITE AND JAMES M. PARKER

*Department of Bacteriology and Immunology, Harvard University Medical School, and the Surgical Research Laboratory, Fifth (Harvard Teaching) Surgical Service, the Boston City Hospital*

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The therapeutic efficacy of sulfanilamide in clinical and experimental infections due to Beta-hemolytic streptococci and certain other bacteria seems to be established. However, the mechanism underlying the action of the drug remains open to conjecture.

Levaditi and Vaisman (1935) suggested that the effectiveness of the drug is due to interference with formation of bacterial capsules. Neither Colebrook and his associates (1936) nor Long and Bliss (1937a) were able to confirm this.

Attention was focussed upon the possible effect of the drug on phagocytosis, when Long and Bliss (1937a) found an unusual increase in phagocytosis of streptococci by both polymorphonuclear and mononuclear leucocytes in peritoneal smears from mice recovering under sulfanilamide therapy. Mellon, Gross and Cooper (1937) studied histological sections of subcutaneous streptococcus lesions in treated and untreated guinea pigs, and were unable to demonstrate that phagocytosis played a primary role in the curative process. Weinberg, Mellon, and Shinn (1937) in work with several hundred mice, found no clear evidence of increased phagocytosis by leucocytes or reticulo-endothelial cells of liver or spleen in drug-treated animals. Long has recently been quoted by Bohlman (1937) as believing that phagocytosis is neither inhibited nor enhanced by the drug.

The possibility of a direct effect of the drug upon bacteria

has attracted considerable attention. Colebrook and his associates (1936) and Long and Bliss (1937 a and b; also Bliss and Long, 1937) have reported a bacteriostatic effect upon streptococci with sulfanilamide in culture media. The failure of Rosenthal (1937) to confirm this was probably due to use of large inocula. The easily demonstrated bacteriostatic action of the drug *in vitro*, together with the findings of Bliss and Long (1937) in connection with experimental infections in mice, seems to throw the weight of evidence in favor of the theory that the action is directly upon the bacteria themselves rather than an effect upon the usual elements involved in defense.

If we accept the view that sulfanilamide acts directly upon bacteria, the question arises as to whether this action is inhibitory or bactericidal (in the sense of sterilization). Helmholtz and his associates (1937), among others, have reported a bactericidal effect with the drug in certain infections of the urinary tract. This effect in urine has been demonstrated *in vitro*. A bactericidal effect in blood has been demonstrated by Colebrook and his associates (1936). This has been confirmed by Finklestone-Sayliss (1937) who found that a bactericidal effect was obtained in de-leucocyted blood as well as in whole blood. Osgood and Brownlee (1938) have reported that sulfanilamide in human marrow cultures effects sterilization when streptococci are added, but these investigators do not believe that the drug alone is able to kill the organisms. Gay and Clark (1937) state that the drug "has no bactericidal effect on the streptococcus when added to nutrient media in the test tube." Bliss and Long (1937) have reported experiments which do not support the belief that sulfanilamide has a bactericidal effect *in vitro*. Nitti and his associates (1937), and Mayer (1937), on the other hand, reported experiments in which the drug apparently brought about sterilization in peptone-glucose water and saline, respectively, when small inocula of streptococci were used.

It is evident from the literature cited above that the question as to whether sulfanilamide acts as a bacteriostatic or as a bactericidal agent is still unsettled.

Although test temperatures were not mentioned in every case,

it is probable that the above-mentioned investigations on the effectiveness of the drug *in vitro* have been carried out at approximately 37°C. As far as we have been able to determine, the effect of the drug at higher temperatures upon streptococci *in vitro* seems to have been overlooked.

The present report contains preliminary data bearing on the bactericidal action of sulfanilamide upon Beta-hemolytic streptococci *in vitro* at an elevated test temperature. Experiments designed to evaluate the factors which condition this bactericidal action will form the basis of subsequent reports.

## MATERIALS AND METHODS

*Strains.* With the exception of B-168, all strains used in the present study had been recently isolated from cases at the Childrens Hospital.[1] These cases included septicemia, meningitis, mastoid, otitis media, peritonitis and scarlet fever infections. Colony variants, determined in accordance with the criteria suggested by Ward and Lyons (1935), included F, M, C and a fourth variant, not as yet described, which we have tentatively designated as the *B variant.* B-168 is the "Richards" strain, used by Colebrook and his associates (1936) and provided through the courtesy of Dr. R. C. Lancefield. Several of the strains used in our tests were highly virulent for mice. The B and M variants developed thick capsules in the PD broth described below.

*Stock cultures.* Cultures in defibrinated horse blood were stored at refrigerator temperature and transferred to fresh horse blood once a month. Under these conditions all four colony variants were found to be stable.

*Test cultures.* Frequent transfer in PD broth was maintained for all cultures tested. The test inoculum consisted of 0.2 cc. of a 1:1000 dilution in broth of a 3- to 5-hour broth culture grown at 37°C. An average initial concentration yielding 5000 colonies per cubic centimeter of test mixture was thus obtained, as determined by blood-agar plate counts.

[1] We wish to thank Dr. Benjamin Carey for his courtesy in supplying the Childrens Hospital strains.

*Sulfanilamide solutions.* Pure para-amino-benzene-sulfonamide, supplied by the Winthrop Chemical Company, was used in our tests. A 0.44 per cent stock solution in distilled water, kept away from light, gave consistent results in repeated tests. 0.2 cc. of stock solution was used to obtain an initial concentration of 20 milligrams per cent in test mixtures. No change in this concentration of drug was detected on completion of tests.

*Peptone-Glucose medium (PD broth)*

| | |
|---|---|
| Neopeptone | 7.0 grams |
| Proteose peptone | 7.0 grams |
| Pfanstiehl peptone | 7.0 grams |
| Sodium chloride | 5.0 grams |
| Potassium phosphate, primary | 1.0 grams |
| Potassium phosphate, secondary | 1.0 grams |
| 20 per cent sodium hydroxide | 3.8 cc. |
| Distilled water | 1000 cc. |

Heat ingredients listed above at 100°C. for 20 minutes. Filter. Add 1.0 gram of glucose, dispense and autoclave for 20 minutes at 112°C. The resulting clear buffered broth should have a pH of approximately 7.5. Abundant and rapid growth from small inocula of many different strains of Beta-hemolytic streptococci has been obtained in this broth. The buffer system prevents the pH from dropping below 6.0 during maximum growth and, thus, death of organisms in old cultures is delayed. Many strains develop capsules in young cultures in this medium. Several strains highly virulent for mice have retained their virulence when cultured in it. PD medium was used throughout the present study: as plain broth; as blood broth, with one drop of defibrinated horse blood added to 9 cc. of PD; as plating medium, with the addition of 1.5 per cent agar.

*Bactericidal test procedure.* Test mixtures were made in round-bottomed rubber-stoppered flasks (25 cc. capacity) in pairs for each strain as follows:

| | *Flask A* | *Flask B (Control)* |
|---|---|---|
| | cc. | cc. |
| Medium (Blood or Broth) | 4.0 | 4.0 |
| Sulfanilamide 0.44 per cent | 0.2 | |
| Saline 0.85 per cent | | 0.2 |
| Culture diluted 1:1000 | 0.2 | 0.2 |

The flasks were attached in a horizontal position to a wheel which was revolved at 10 R.P.M. in an incubator. As each flask rotated, its contents were continuously spread and rolled over the glass surface, which insured satisfactory mixture throughout the test period. The initial concentration of each test mixture was determined by making a similar mixture and immediately transferring 1.0 cc. through a series of 9 cc. blood broth dilution blanks. The bacterial concentration was expressed as the $\log_{10}$ of the highest dilution of test mixture in blood broth which showed growth after 72 hours incubation at 37°C. After incubation of mixtures for test period, bacterial concentrations were again determined by quantitative dilution in tubes of blood broth. Growth in blood broth tubes was checked by loop streaks on blood agar plates.

*Criteria for sterility in test mixtures.* Test mixtures were considered to be sterile when growth was not obtained in the following subcultures incubated at 37°C. for 72 hours: (*a*) loopful streaked on blood agar plate; (*b*) blood agar pour plate culture of 0.5 cc. of undiluted test mixture; (*c*) 1.0 cc. of test mixture added to 9 cc. of PD blood broth.

## RESULTS

Preliminary tests had convinced us that a concentration of as much as 100 mgm. per cent sulfanilamide in whole blood, or broth, at 37°C. failed to sterilize an inoculum of certain strains of Beta-hemolytic streptococci; when an initial concentration in test mixtures of 1,000 to 10,000 bacteria per cubic centimeter was used.

During the course of some work carried out at the Harvard School of Public Health, a year ago, it was observed that many freshly isolated strains of hemolytic streptococci grew rapidly and abundantly at 40°C. A few trial mixtures of sulfanilamide and streptococci in blood were tested at elevated temperatures and what appeared to be a bactericidal action, due to the drug, was consistently obtained at 40°C. with 20 mgm. per cent sulfanilamide in horse blood during 48 hours incubation. The bactericidal effect was not obtained at 37°C. These observations led to the present study.

The first part of our work was directed to determine if the bactericidal action noted above was confined to the particular strains used. Accordingly, the bactericidal test technique, described elsewhere in this paper, was used with 36 different strains freshly isolated from various human infections. In all cases, sterilization of the test inoculum, in normal defibrinated horse blood containing 20 mgm. per cent of the drug, was obtained during 48 hours incubation at 40°C. Control mixtures, without the drug, simultaneously incubated, showed complete hemolysis of blood and abundant growth of bacteria at the end of the test period. Blood freshly drawn gave the same result as that which had been refrigerated for as long as two weeks.

We have fully appreciated the fact that it is often extremely difficult to distinguish inhibitory and bactericidal action. Subcultures may fail to show growth, not because test mixtures are sterile, but because of continuing inhibition. The strongest concentration of sulfanilamide used in our tests was 20 mgm. per cent. Subcultures made by transferring 1.0 cc. of test mixture into 9 cc. of PD blood broth diluted the test concentration of drug 1:10, or, to 2 mgm. per cent. Therefore, tests were made to determine whether 2, or more, mgm. per cent of sulfanilamide would inhibit a small number of viable bacteria under subculture conditions, i.e., in blood broth incubated at 37°C. To one series of PD blood broth blanks sulfanilamide was added to a final concentration of 2.5 mgm. per cent. A culture of streptococci was then diluted in PD broth out to $10^{-8}$. The sulfanilamide-blood-broth and a control blood-broth series were then inoculated with the culture dilutions containing decreasing numbers of bacteria and all tubes were incubated at 37°C. for 48 hours. In no case did 2.5 mgm. per cent sulfanilamide prevent abundant growth from an inoculum yielding a single colony, as determined by plate counts. Similar tests were made with several different strains. In all cases, the smallest bacterial inoculum grew out equally well, with or without the drug. Moreover, it was found that as much as 20 mgm. per cent sulfanilamide did not prevent the growth of small inocula in PD blood broth at 37°C. Additional evidence was obtained by re-inoc-

ulating test subculture tubes which appeared to be sterile with a small number of viable bacteria (from 1 to 10 colonies). The growth resulting from this second inoculum was interpreted as meaning that growth would also have resulted from the test mixture inoculum if it had contained any viable bacteria. We believe that the foregoing results justify our assumption that

TABLE 1

*Effect of 20 mgm. per cent sulfanilamide upon Beta-hemolytic streptococci in normal defibrinated horse blood*

| STRAIN | INITIAL BACTERIAL CONCENTRATION | BACTERIAL CONCENTRATION AFTER 48 HOURS | | | |
|---|---|---|---|---|---|
| | | Sulfanilamide-blood | | Blood | |
| | | 40°C. | 37°C. | 40°C. | 37°C. |
| F-20 | 4 | Sterile | 7 | 8 | 9 |
| F-24 | 4 | Sterile | | 7 | |
| F-112 | 4 | Sterile | | 7 | |
| F-115 | 4 | Sterile | | 9 | |
| M-1 | 4 | Sterile | | 8 | |
| M-146 | 4 | Sterile | 6 | 7 | 8 |
| M-210 | 3 | Sterile | | 8 | |
| B-4 | 4 | Sterile | | 9 | |
| B-9 | 4 | Sterile | | 8 | |
| B-168 | 4 | Sterile | | 8 | |
| B-171 | 4 | Sterile | 6 | 8 | 9 |
| B-208 | 4 | Sterile | | 9 | |
| C-17 | 3 | Sterile | | 8 | |
| C-201 | 4 | Sterile | 8 | 8 | 9 |
| C-241 | 5 | Sterile | | 7 | |
| C-246 | 3 | Sterile | | 8 | |

Bacterial concentrations were determined by diluting 1.0 cc. of each test mixture with blood broth 1:10, 1:100, 1:1000, etc. Figures above indicate the logarithm$_{10}$ of the highest dilution of test mixture in blood broth in which growth occurred during incubation at 37°C. for 72 hours.

failure to obtain subculture growth meant sterilization, not merely inhibition, under our test conditions. Therefore, we have applied the term "bactericidal" to the effect obtained with sulfanilamide in our tests.

The results of bactericidal tests with 16 strains are given in table 1. Bacterial concentrations in test mixtures are expressed as the logarithm$_{10}$ of the highest dilution in blood broth showing

growth after 72 hours incubation at 37°C. Occasional plate counts showed that we were using an initial concentration of bacteria yielding about 5,000 colonies per cubic centimeter. In blood without sulfanilamide this initial concentration usually increased, during incubation at 40°C., to about 100,000,000 per cc., i.e., a concentration such that 1.0 cc. could be diluted to $10^{-8}$ and still show growth by our dilution method of subculture. Blood-bacteria mixtures to which sulfanilamide had been added, showed no hemolysis and apparently contained no viable bacteria, as determined by application of our criteria for sterility. It will be seen from the results recorded in table 1 that 20 mgm. per cent sulfanilamide was bactericidal at 40°C. for all 16 strains tested, and that this effect was not obtained with 4 strains similarly tested at 37°C.

The action of the drug in plain PD broth was then tested. The same bactericidal test method was used. The results, as recorded in table 2, indicate bactericidal action at 40°C., an effect which was lacking at the lower temperature. Four strains were used in these tests: F-20, four times; M-146, once; B-171, five times; C-201, twice.

The experiments outlined above showed that a bactericidal effect could be demonstrated with 20 mgm. per cent sulfanilamide under conditions which were otherwise favorable for abundant bacterial growth. However, the method used failed to illuminate the nature of the bactericidal action involved. Therefore, growth curves were plotted to determine the beginning and progress of bactericidal action to the point of sterility.

*Growth curve procedure.* Mixtures of bacteria and drug in PD broth, similar to those described above, were similarly incubated. 0.2 cc. samples removed at frequent intervals were diluted 1:10 in 1.8 cc. of broth, further quantitative dilution being carried out by transferring 1.0 cc. of this 1:10 dilution through 9 cc. blood broth blanks. 1.0 cc. of at least two dilutions was plated out in blood-agar pour plates.

Results of preliminary growth-curve tests with a large number of mixtures confirmed the observations previously noted. An inhibitory effect, due to sulfanilamide, on all strains tested, was

obtained at 36°C. As the test temperature was raised to 39°C., this inhibitory effect increased. Not until the test temperature exceeded 39°C. was a bactericidal effect (sterilization) obtained with the inoculum used.

In table 3 are recorded experiments which show the effect of 20 mgm. per cent of sulfanilamide upon the multiplication of four strains of streptococci in PD broth at 37° and 40°C. In

TABLE 2

*Effect of 20 mgm. per cent sulfanilamide upon Beta-hemolytic streptococci in PD broth*

| STRAIN | INITIAL BACTERIAL CONCENTRATION | BACTERIAL CONCENTRATION AFTER 48 HOURS | | | |
|---|---|---|---|---|---|
| | | Sulfanilamide-broth | | Broth | |
| | | 40°C. | 37°C. | 40°C. | 37°C. |
| F-20 | 4 | Sterile | 6 | 9 | 9 |
| F-20 | 4 | Sterile | | 9 | |
| F-20 | 5 | Sterile | | 9 | |
| F-20 | 4 | Sterile | | 9 | |
| M-146 | 4 | Sterile | 6 | 8 | 8 |
| B-171 | 6 | Sterile | | 9 | |
| B-171 | 4 | Sterile | 7 | 9 | 8 |
| B-171 | 3 | Sterile | | 8 | |
| B-171 | 5 | Sterile | | 8 | |
| B-171 | 3 | Sterile | | 8 | |
| C-201 | 5 | Sterile | | 8 | |
| C-201 | 4 | Sterile | 7 | 8 | 8 |

Bacterial concentrations were determined by diluting 1.0 cc. of each test mixture with blood broth 1:10, 1:100, 1:1000, etc. Figures above indicate the logarithm$_{10}$ of the highest dilution of test mixture in blood broth in which growth occurred during incubation at 37°C. for 72 hours.

these tests 0.2 cc. of a 1:1000 broth dilution of a 4- to 6-hour broth culture was added to 4.2 cc. of broth containing enough sulfanilamide to give a final drug concentration of 20 mgm. per cent. A control flask containing no drug was similarly inoculated. All flasks were rotated to maintain conditions under which our previous results had been obtained. Samples removed at intervals of three hours were plated in blood agar, after suitable dilution. In table 3 plate counts are expressed as the

logarithm$_{10}$. These results are presented as time-action curves in charts 1, 2, 3, and 4.

The curves in chart 1 show that growth in the plain broth controls was characterized by multiplication at a rapid rate during the first twelve hours, reaching a growth level which remained practically unchanged up to the thirtieth hour of in-

TABLE 3

*Effect of 20 mgm. per cent sulfanilamide upon the multiplication of Beta-hemolytic streptococci in PD broth at 37 and at 40°C.*

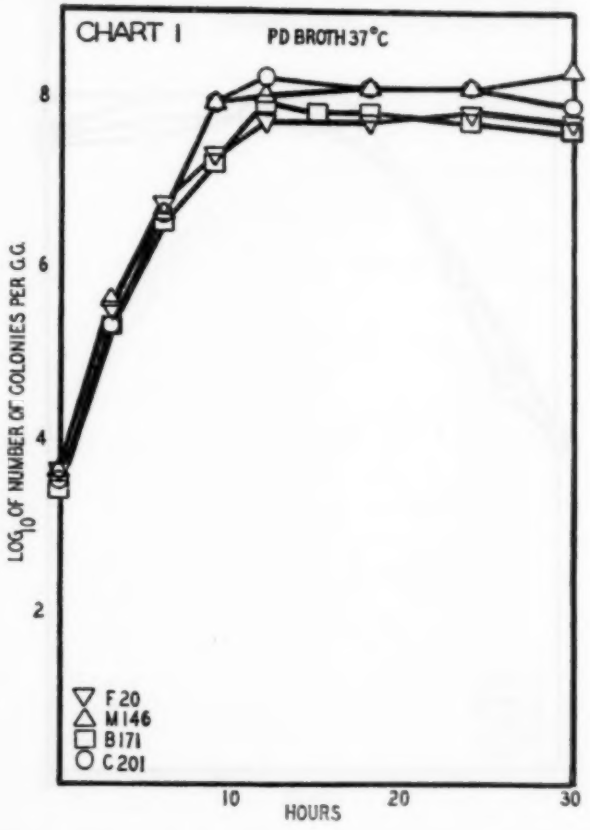
| STRAIN | MEDIUM | TEMPERATURE | HOURS | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 30 |
| | | °C. | | | | | | | | | | | |
| F-20 | Sulf.-Broth | 37 | 3.6 | 5.3 | 6.1 | 6.5 | 6.8 | | 6.7 | | 6.3 | | 5.6 |
| | Broth | 37 | 3.6 | 5.5 | 6.7 | 7.3 | 7.7 | | 7.7 | | 7.8 | | 7.7 |
| | Sulf.-Broth | 40 | 3.7 | 4.9 | 5.5 | 5.5 | | 4.5 | 3.8 | | 2.6 | | Sterile |
| | Broth | 40 | 3.7 | 5.1 | 5.9 | 6.9 | | 7.5 | 7.8 | | 7.8 | | 7.4 |
| M-146 | Sulf.-Broth | 37 | 3.6 | 5.3 | 5.9 | 6.2 | 6.0 | | 5.9 | | 5.9 | | 6.2 |
| | Broth | 37 | 3.6 | 5.6 | 6.6 | 7.9 | 8.0 | | 8.1 | | 8.1 | | 8.3 |
| | Sulf.-Broth | 40 | 3.8 | 4.8 | 5.1 | 5.3 | | 5.1 | 4.6 | | 1.9 | | Sterile |
| | Broth | 40 | 3.8 | 4.9 | 5.9 | 6.8 | | 7.8 | 7.8 | | 7.8 | | 7.8 |
| B-171 | Sulf.-Broth | 37 | 3.4 | 5.0 | 5.7 | 6.2 | 6.6 | 7.0 | 7.0 | | 7.0 | | 6.9 |
| | Broth | 37 | 3.4 | 5.3 | 6.5 | 7.2 | 7.9 | 7.8 | 7.8 | | 7.7 | | 7.6 |
| | Sulf.-Broth | 40 | 3.8 | 4.3 | 4.4 | 4.1 | 2.3 | 1.0 | 0 | | 0 | | Sterile |
| | Broth | 40 | 3.8 | 4.3 | 5.7 | 6.9 | 7.8 | 7.8 | 7.6 | | 7.5 | | 7.3 |
| C-201 | Sulf.-Broth | 37 | 3.5 | 5.2 | 6.1 | 6.3 | 6.5 | | 6.6 | | 7.0 | | 7.0 |
| | Broth | 37 | 3.5 | 5.3 | 6.6 | 7.9 | 8.2 | | 8.1 | | 8.1 | | 7.9 |
| | Sulf.-Broth | 40 | 3.7 | 4.4 | 5.0 | 5.1 | 4.1 | 3.0 | 0 | | 0 | | Sterile |
| | Broth | 40 | 3.7 | 4.5 | 5.9 | 6.8 | 7.4 | 7.4 | 7.6 | | 7.4 | | 7.2 |

Figures in table above indicate bacterial concentrations per cubic centimeter expressed as the logarithm$_{10}$ of pour plate counts.

cubation. Raising the test temperature to 40°C. slowed down the optimal rate of multiplication in plain broth controls. Also, with 3 of the 4 strains, the maximal bacterial concentration at this temperature was at a level slightly lower than that reached at 37°C., as may be seen by comparing corresponding curves in charts 1 and 2. The absence of a significant lag period in any

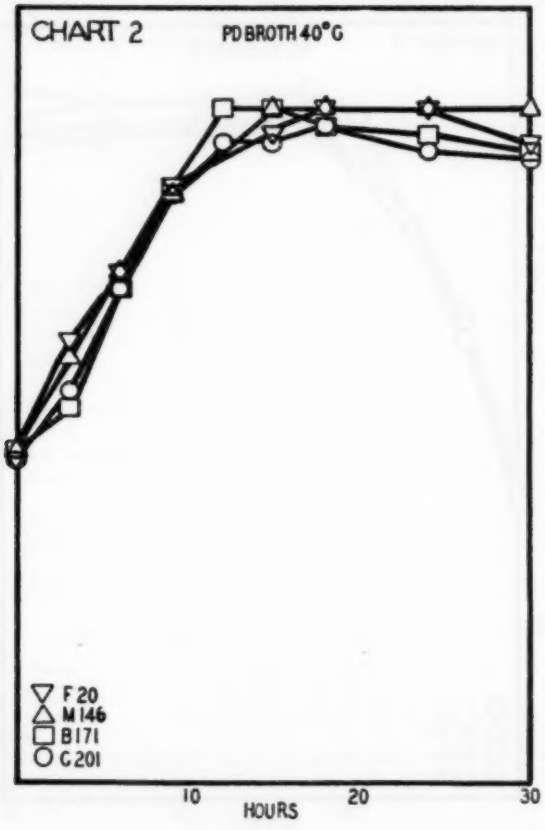
of the test mixtures is probably due to the fact that young cultures were always used as a source of test inocula.

Charts 3 and 4 present growth curves obtained when bacteria were added to broth containing 20 mgm. per cent sulfanilamide,



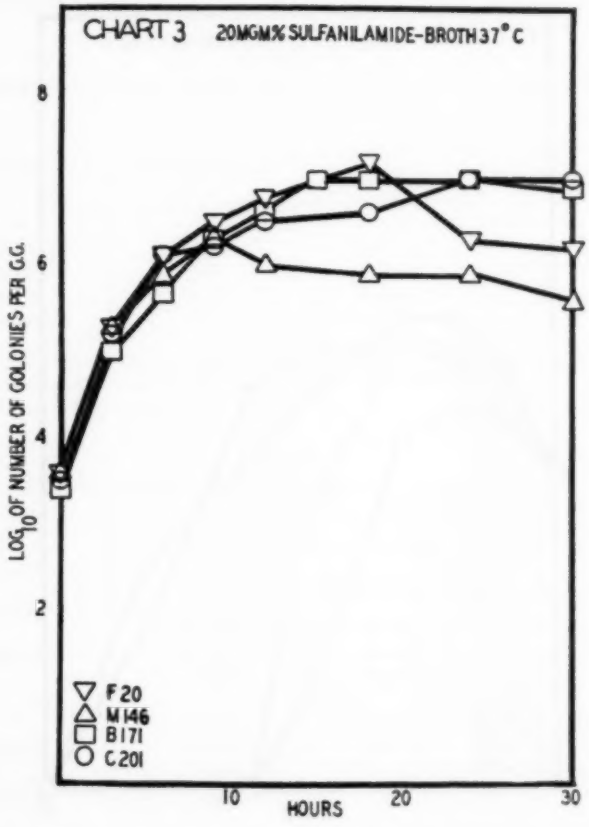

incubation being carried out at 37° and at 40°C. respectively. It is evident that at both temperatures the drug had little or no effect upon bacterial multiplication during the first three hours. This delay in the action of the drug is typical. Repeated tests

with many strains have always shown a delay in the action of sulfanilamide lasting for about three hours. To date, we have been unable to determine the factors responsible for this continued multiplication of the streptococci in the presence of



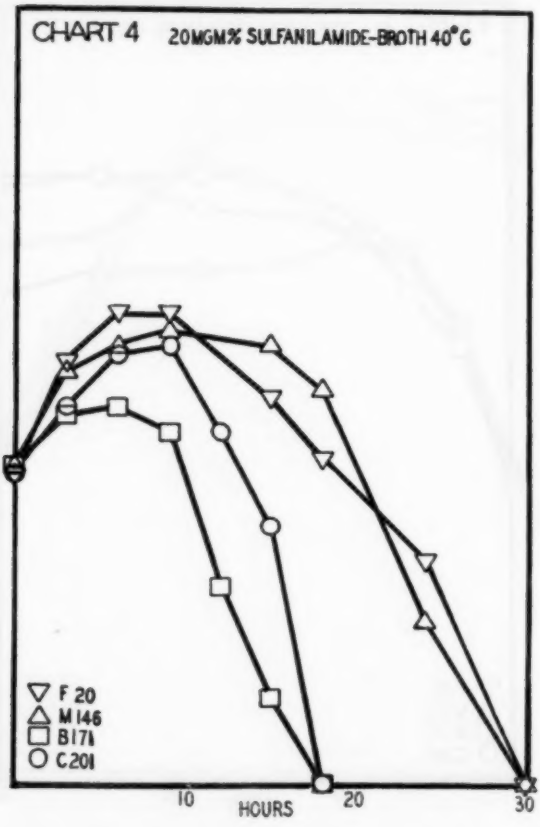

sulfanilamide during the initial state of incubation. Between the third and sixth hour, the beginning of an inhibitory effect upon all four strains, at both test temperatures, is apparent. This inhibitory effect has continued throughout the test period at 37°C. (Chart 3). At 40°C., de-celeration of growth rate has

continued up to the ninth hour followed by a progressive decrease in bacterial concentration to the point of sterility: in eighteen hours with the strains B-171 and C-201; in thirty hours with strains F-20 and M-146 (Chart 4).




Results obtained under our test conditions had clearly indicated that a bactericidal effect with 20 mgm. per cent sulfanilamide could be demonstrated at 40°C. We then tested a series of mixtures containing 10 mgm. per cent of the drug and obtained the results shown in table 4. It may be seen that test mixtures

with an initial concentration yielding approximately 10,000 colonies per cubic centimeter were sterilized by 10 mgm. per cent sulfanilamide in 30 hours at 40°C. No attempt was made during the present study, to determine the minimal bactericidal




concentration of sulfanilamide for various sizes of initial streptococcal concentrations.

The effect of 20 mgm. per cent sulfanilamide, at 40°C., upon three different initial concentrations of each of two strains is shown in table 5. These results indicate that strain B-168 is more

susceptible than B-170 to the bactericidal action of the drug. B-168 is our laboratory number for the "Richards" strain used by Colebrook and his associates (1936).

Quantitative relationships between drug and bacteria in terms of bactericidal effect at 40°C., together with the question of

TABLE 4

*Bactericidal effect of 10 mgm. per cent sulfanilamide upon Beta-hemolytic streptococci in PD broth at 40°C.*

| STRAIN | INITIAL BACTERIAL CONCENTRATION | BACTERIAL CONCENTRATION AFTER 30 HOURS | |
|---|---|---|---|
| | | Sulfanilamide-broth | Broth |
| F-20 | 4.1 | Sterile | 7.7 |
| M-146 | 4.1 | Sterile | 7.8 |
| B-171 | 3.4 | Sterile | 7.0 |
| B-171 | 4.1 | Sterile | 7.8 |
| C-201 | 4.2 | Sterile | 7.2 |

Bacterial concentrations determined by plating 1.0 cc. of test mixtures in blood agar pour plates after making required dilutions in broth. Figures above indicate the $\log_{10}$ of plate counts.

TABLE 5

*Bactericidal effect of 20 mgm. per cent sulfanilamide upon large and small inocula of two strains of Beta-hemolytic streptococci in PD broth at 40°C.*

| STRAIN | INITIAL BACTERIAL CONCENTRATION PER CC. | GROWTH AFTER 48 HOURS INCUBATION | |
|---|---|---|---|
| | | Sulfanilamide-broth | Broth |
| B-168 | 350,000 | Sterile | ++++ |
| | 35,000 | Sterile | ++++ |
| | 3,500 | Sterile | ++++ |
| B-170 | 170,000 | ++++ | ++++ |
| | 17,000 | Sterile | ++++ |
| | 1,700 | Sterile | ++++ |

variation in degree of strain susceptibility will be dealt with in a subsequent report.

## COMMENT

The mode of action of sulfanilamide, *in vitro* as well as *in vivo*, has remained obscure. Several investigators (Colebrook

*et al.*, 1936; Long and Bliss, 1937 a and b; Bliss and Long, 1937; Finklestone-Sayliss, 1937) have concluded that the drug's action is directly upon the bacteria, but there has been incomplete agreement as to whether this action is truly bactericidal or merely inhibitory in nature. Our studies have been in connection with the effect of sulfanilamide *in vitro* only. Under these conditions, we believe that the effect obtained with the drug, on hemolytic streptococci, will depend principally upon the temperature at which the test is run.

The fact that bactericidal action had been observed with the drug in whole blood at 40°C., when only inhibition could be demonstrated at 37°C., led us to conclude that the temperature at which bacteria were subjected to action of the drug determined whether sterilization or inhibition would result. However, we realize that it is difficult to draw definite conclusions from results with a test mixture which contains not only drug and bacteria, but leucocytes and serum as well.

In plain PD broth, on the other hand, it seems to us that there can be no question of bactericidal action on the part of any element other than the drug itself. With such a mixture we are obviously dealing only with bacteria multiplying in an environment which includes nutritive elements, metabolites and drug. Under these conditions, it appears that temperature is the determining factor. Although we have repeatedly verified our bactericidal results at 40°C., we have, thus far, been unable to obtain data which will explain just why the streptococci are killed by the drug at 40°C. when they are merely inhibited at 37°C. under conditions otherwise similar.

The significance of a bactericidal action with sulfanilamide at 40°C. *in vitro* should not be overlooked. Bactericidal action with this drug has been demonstrated by us only at elevated temperatures corresponding to those associated with high fevers. This suggests a correlation with successful clinical treatment of patients with severe hemolytic streptococcus infections involving high temperatures. Hence, in rapidly successful sulfanilamide therapeusis, it may be that the invading bacteria have been exposed, at times, to the action of the drug at temperatures

similar to those at which bactericidal action has been demonstrated *in vitro*. As far as we have been able to determine, there has been but one report on the combined use of hyperthermotherapy and sulfanilamide. Ballenger, Elder and McDonald (1937), in a clinical note have reported successful treatment of a few cases of gonococcic infection with the administration of an artificial fever of 103° to 104°F. combined with sulfanilamide. It is, perhaps, significant that patients chosen for this combined treatment were those who had failed to respond to either treatment alone.

The significance of our observations in connection with making comparative tests on sulfanilamide and related compounds, *in vitro*, is obvious.

## CONCLUSIONS

Bactericidal action upon thirty-six strains of Beta-hemolytic streptococcus recently isolated from human infections has been demonstrated with 20 mgm. per cent sulfanilamide *in vitro* at 40°C. in peptone-glucose broth and in whole blood. Preliminary results indicate that this streptococcidal action can be demonstrated *in vitro* with lower concentrations of sulfanilamide.

Under our experimental conditions, bactericidal action due to sulfanilamide could not be demonstrated at test temperatures lower than 39°C.

Experimental evidence indicates that the action of sulfanilamide *in vitro* is delayed for at least three hours.

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# A SYNTHETIC MEDIUM FOR THE CULTIVATION OF C. DIPHTHERIAE[1]

J. HOWARD MUELLER

*Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts*

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It has been shown by Pappenheimer and Johnson (1936), Pappenheimer (1936), and Johnson, Pappenheimer and Robinson (1938), that diphtheria toxin containing 50 to 60 $L_f$ doses per cubic centimeter may regularly be produced on a medium of simple chemical composition. That this is by no means the upper limit of toxin production is demonstrated by the fact that the same strain of Park 8 produces as much as 100 $L_f$ per cubic centimeter on a pig-stomach digest medium, Taylor (1935). It is therefore probable that the "synthetic" medium can be improved to a point where it will equal or excell the empirical one in toxin production.

The simplified medium used by Pappenheimer and co-workers is, in a general way, based on work which has been going on for some time in this laboratory concerning growth requirements of the diphtheria bacillus as a part of a general program of study of nutritive requirements of pathogenic organisms. Many of the results obtained have already been presented (Mueller and collaborators 1933–1938), and since it is now possible to complete the listing of substances essential to very heavy growth of the strain which has been most intensively studied, thus opening the way for further work on toxin production, it may not be out of place to summarize very briefly the more important points which have been established.

[1] Through the courtesy of various members of its Faculty, part of these experiments were carried out at the Dalhousie University Medical School, Halifax, Nova Scotia, during the summer of 1937.

The plan of approach was to start with a known, suitable medium (meat-extract broth) and attempt to isolate or identify the chemical substances contributing to its usefulness. Fortunately, for the sake of comparison of results over a period of time, it was thought best to adopt a relatively exact method for estimating growth, and this was done by the determination of nitrogen in the centrifuged and washed bacteria from 10 cubic centimeter quantities of medium after an optimal period of incubation. Most strains of the diphtheria bacillus yield in this way from 1 to 2 milligrams of bacterial nitrogen on nutrient bouillon.

The broth was considered to be composed of two classes of compounds, (*a*) "peptones," or protein split-products of varying degree of complexity, and (*b*) muscle extractives. It was first shown that a protein such as casein, thoroughly hydrolyzed by strong acid to the amino acid stage, and in some cases reinforced by tryptophane (which is destroyed by this procedure), could be substituted for the peptone. Commercial meat extract was used as the source of extractives. Neither the meat extract alone, nor the hydrolyzed protein alone supported good growth; together, particularly when some enriching substance, e.g., maltose, ethyl alcohol, glycerol or lactic acid was added, quite satisfactory growth was obtained.

It was then possible to determine, in the case of several strains of the organism, which of the amino acids were essential, and in what quantity. Differences appeared among the various strains, although certain amino acids, such as cystine, glutamic or aspartic[2] acid, methionine and valine were required by all or most strains.

The meat extract fraction was next examined. It was found, as might have been anticipated, that certain essential inorganic materials were supplied by it, i.e., Mg, K, and $PO_4$. In addition, however, organic substances were involved which were effective in extremely small amounts. Various liver extract fractions

[2] Aspartic acid appeared not to be used by the first strain which was reported ("HY"). Later work indicated that some strains prefer glutamic acid, others aspartic acid, and others do best on a mixture of the two.

were actually used at this stage, by-products from preparations of the pernicious anemia curative material, obtained through the courtesy and cooperation of Dr. Y. Subbarow of the Department of Biological Chemistry, Harvard University Medical School and of the Lederle Laboratories, Inc.

With this material it was shown that the essential organic materials could be separated into two fractions by ether extraction from acid solution. The ether-soluble material, found also to occur in high concentrations in the urine of herbivors, was isolated from cow's urine and identified as pimelic acid, a substance which had previously not been known to have biological importance. The material not extracted by ether was shown to contain two essential compounds. These were obtained from liver extract by esterification and acetylation, and in this form, careful vacuum distillation effected the separation. One was isolated in pure crystalline condition and identified as nicotinic acid, which had just been shown by Knight (1937) to be essential to the growth of the staphylococcus. The other, although not actually isolated, had properties which led to the belief that it might be $\beta$-alanine, which had previously been shown by Williams and Rohrman (1936) and by Miller (1936) to be essential to yeast growth. This proved to be the case, and the three substances pimelic acid, nicotinic acid and $\beta$-alanine, together with suitable amino acids, inorganic salts, and sodium lactate, yielded growth of approximately 2.25 mgm. bacterial nitrogen per 10 cubic centimeter of medium. It is on substrates of this type that Pappenheimer and co-workers have been able to produce potent toxin with a strain of the Park 8 organism when care was taken to remove excess iron, which occurs in all the materials used as an impurity.

While such growth was good, it was not as heavy as had been obtained with more impure materials; 3.5 mgm. N had frequently been observed, and both from the theoretical side of the matter, and the practical one of perhaps increasing the toxin yield, the work has been continued to learn the conditions under which better growth can be obtained.

It may at this point be again emphasized that there are many

differences in nutritive requirements between strains of *corynebacterium diphtheriae*. Even between strains of supposed Park-Williams no. 8 obtained from various laboratories, these differences are striking, and involve amino acids, sources of energy, e.g., lactic acid, glycerol, etc., and the accessory substances. Thus, while β-alanine and nicotinic acid seem to be required by practically all strains, pimelic acid increases the growth of only about half the strains so far tried and is without effect on the others. Moreover, two strains, both Park 8, one from Albany, N. Y., the other from Utrecht, Holland, have been encountered, which fail to grow on such media, and evidently have some further or different requirements. Whether all these differences within what is supposed to be a single strain represent variation over a period of many years, or whether other organisms have in some instances been substituted for the Park 8, there is at present no means of knowing. It is important that it be realized, however, that the medium to be described in this paper will not produce similar results with all strains of the organism.

The work which is to be described has been carried out on our "Allen" strain (not a Park 8), which has been used and described in an earlier paper. It gives a positive virulence test in guinea pigs, but produces practically no toxin, under conditions favorable to toxin production with the Park 8 used at the Massachusetts State Antitoxin Laboratory. It has the great advantage of growing rather more rapidly than the Park 8, making possible an earlier assessment of experimental results.

It is now possible to obtain approximately 9.0 mgm. bacterial nitrogen with this strain from 10 cc. of medium. The increase from the 2.25 level originally obtained, to the present one, has been, the result of increasing the quantity of two of the materials used previously,—*cystine* and *lactic acid*, and the addition of traces of suitable *heavy metals*,—iron, manganese, copper and zinc. The experiments may be discussed under these three headings but since the final result depends upon the proper adjustment of all three, it is naturally impracticable to describe experiments under each heading involving only that one material

or group. In the interest of brevity, therefore, only a few crucial experiments will be given in detail, which will illustrate the effect of all the substances concerned.

### CYSTINE

Although 1.0 mgm. of cystine in 10 cc. of medium was more than sufficient for maximal growth when meat or liver extract was used in the medium, with purified extractive substances it became, at that concentration, the limiting factor. With all other components present in optimal amount, experiments in which increasing quantities of cystine are used show maximum growth to require from 6.0 to 8.0 mgm. of the amino acid in 10 cc. At a concentration of 8.0 mgm. or more, there is considerable danger of separation of the cystine in crystalline form. Up to that point the other amino acids are quite able to hold it in solution, in spite of its low solubility in pure water. One merely adds it as the hydrochloride to the other components of the medium, adjusting the reaction to pH 7.6 with NaOH only when everything else is added and the mixture has been diluted nearly to its final volume. If it is permitted to crystallize out, it will not redissolve during autoclaving, and the medium cannot be used.

It is possible that this increased requirement for cystine represents an additional function of the cystine in replacing some factor present in the tissue extract, although no direct evidence on the point has been obtained. There is no doubt that a portion of its action is due to an effect on the oxidation-reduction potential of the medium, but this is probably not the full explanation. In view of the simultaneous requirement for methionine shown by all strains so far examined, the sulfur metabolism of the organism must be complex.

### LACTIC ACID

The substance "lactic acid" as commonly purchased, is a syrupy solution, marked as containing 85 per cent or somewhat more of the pure chemical. If this commercial material be added to a culture medium, the pH adjusted and the medium auto-

claved, it will invariably be found to have turned acid. In most of this work, a sodium lactate solution has been prepared by diluting the commercial acid with about an equal volume of water, adding phenol red, and neutralizing with 35 to 40 per cent NaOH to distinct alkalinity. The very hot solution is then boiled for a few minutes, adding more NaOH drop by drop when the purple color begins to turn red, until the color is permanent. The solution is then diluted to four times the original volume of 85 per cent lactic acid. This solution keeps indefinitely and is added to media as required. The explanation for the failure of the acid to be sharply neutralized probably lies in the fact that the commercial material contains a considerable amount of lactic anhydride, which is rather slowly converted to the acid on dilution and neutralization. Recently, however, owing to anomalous results in growth with two new lots of lactic acid, it seemed necessary to inquire more carefully into the nature of the material marketed as "lactic acid."

The commercial product is prepared by fermentation. Certain organisms are known to produce mostly *d*-lactic acid, the form which occurs in muscle, and known also as "sarcolactic" acid. Other organisms form the laevo isomer. One would expect, therefore, in commercial preparations that both *d*- and *l*-lactic acid would occur, but not necessarily in equivalent proportions, unless the material were completely racemized at some step in the refinement.

Two different bottles of commercial lactic acid were therefore examined for optical rotation, and each found to have a slight effect on polarized light. In order to insure the absence of metals, ethyl lactate was prepared from each of these and also from a third specimen secured in the meantime. The three ethyl lactates boiling at about 70° under 36 mm. pressure gave the following optical rotations, in a 1 dm. tube: I, −6.22°; II, +1.93°; III, −6.47°. Beilstein gives for *d*-lactic $[\alpha]_D^{14}$ - 10.33°. Other sources give slightly larger figures up to 13° or even 14°. It is to be noted that the direction of the rotation of the acid is reversed with the ester.

A specimen of d-calcium lactate from the U. S. Department of Agriculture yielded a preparation of ethyl lactate showing a rotation of -8.62°, indicating that some racemization took place during the preparation.

Taking the figure −10.33 as 100 per cent optical activity for the substance, and without making the slight corrections for temperature and density, it may be calculated that the various specimens of lactic acid had approximately the following composition: I, 80 per cent *d* and 20 per cent *l*; II, 41 per cent *d* and 59 per cent *l*; III, 81 per cent *d* and 19 per cent *l*; U. S. Dept Agr., 92 per cent *d* and 8 per cent *l*.

The ethyl lactates were converted to sodium lactate by adding water and NaOH, with warming until a permanent alkaline reaction was reached, and were then subjected to vacuum distillation to remove the alcohol.

Using a casein hydrolysate medium like formula *A*; (see final section) but containing lactates from the above three different commercial specimens, and also one from pure *d*-lactic acid, the following results were obtained: (1) Control with lactate I, equivalent to 0.15 gram lactic acid, 7.02 mgm. N. (2) Control with lactate II, equivalent to 0.15 gram lactic acid, 5.57 mgm. (3) Control with lactate III, equivalent to 0.15 gram lactic acid, 7.38 mgm. (4) Control with pure *d*-lactic acid, 0.15 gram, 8.10 mgm.

Similar results were obtainable with great regularity, the amount of growth always quite parallel with the proportion of the dextro acid present.

A further experiment, designed to test directly the utilization of *l*-lactic acid, was then carried out. This material is not commercially available, and if it is not utilized by the diphtheria bacillus, it seemed probable that it could be isolated from cultures grown on commercial lactic acid from which the *d* form had been used up. For this purpose, sample II (above) of lactic acid, containing a slight excess of *l* over *d* was used.

Two liters of the casein hydrolysate medium (formula A) were prepared, containing 1.75 per cent of this commercial acid, steril-

ized in several Kolle flasks and inoculated with the Allen strain. After four days' incubation, flasks and contents were steamed, and the bacterial growth centrifuged out. The supernatants were concentrated *in vacuo* to about $\frac{1}{5}$ the volume, strongly acidified with $H_2SO_4$ and extracted with butyl alcohol. The alcohol was then extracted with NaOH solution and again used for a second extraction of the medium. In all, six double extractions were carried out, and then the combined NaOH extracts, containing the lactic acid as well as other materials, were concentrated in vacuum, acidified with $H_2SO_4$ and extracted continuously with ether. Evaporation of the ether yielded a strongly acid syrup, from which some crystalline material, identified as succinic acid, separated. The remainder was converted to the zinc salt and fractionally crystallized. After removal of an initial crop of zinc *dl* lactate, an abundant crop of material was obtained, which after two recrystallizations was found to be pure zinc *l* lactate (correct optical rotation and water of crystallization.)

This material, freed from zinc as described below, was tested for its effect on growth of the test organism, using again a medium of formula *A*, but containing no lactic acid. For comparison, a similar set to which *d*-lactic acid was added is also given. In each case, of course the compound is added as the Na salt.

| | gram | mgm. N |
|---|---|---|
| 1. Control + | | 2.29 |
| 2. Control + *l*-lactic acid | 0.025 | 1.50 |
| 3. Control + *l*-lactic acid | 0.05 | 2.60 |
| 4. Control + *l*-lactic acid | 0.1 | 3.06 |
| 5. Control + *l*-lactic acid | 0.125 | 3.12 |
| 6. Control + *l*-lactic acid | 0.15 | 2.82 |
| 7. Control + *l*-lactic acid | 0.175 | 2.60 |
| 8. Control + *d*-lactic acid | 0.025 | 4.49 |
| 9. Control + *d*-lactic acid | 0.05 | 5.70 |
| 10. Control + *d*-lactic acid | 0.1 | 7.76 |
| 11. Control + *d*-lactic acid | 0.125 | 8.80 |
| 12. Control + *d*-lactic acid | 0.15 | 8.26 |
| 13. Control + *d*-lactic acid | 0.175 | 9.12 |

In spite of the irregularity in tubes 2, 11 and 12 the results are perfectly clear-cut. The moderate increase shown with *l*-lactic

acid may be due either to slight racemization during autoclaving, or to a direct effect on the part of the organism.

It is clear from all the evidence that, in the case of this strain, utilization of lactic acid is pretty definitely limited to the *d* form occurring naturally in body tissues. Whether this will hold for other strains of *C. diphtheriae* remains to be determined. The amount of growth appears to increase up to the point at which the concentration becomes inhibitory, perhaps through osmotic effects or an excess of Na. This concentration (the equivalent of approximately 2.0 per cent lactic acid) seems to be independent of the optical form of the material and consequently, the greater the proportion present in the *l* form, the less is available for growth. The use of a fairly pure *d*-lactic acid, therefore, leads to much higher levels of growth than is possible with the commercial acids.[3]

## HEAVY METALS

At a very early stage of these studies it was recognized that significant amounts of phosphorus were furnished to the medium by meat extract. Later, with the use of charcoal-adsorbed material, potassium and magnesium deficiencies appeared, and were overcome by changing the formula of the salt mixture to contain quantities within the optimal range of concentration. Iron salts, included in the earlier media, had been omitted when the work of Pappenheimer and Johnson showed this element already to be present in the reagents in sufficient concentration to inhibit toxin formation.

The substitution of $\beta$-alanine, nicotinic and pimelic acid in minute quantities for tissue extract of course withdrew a very important source of mineral impurity and made necessary a

[3] The zinc salt of *d*-lactic acid, a generous supply of which was obtained from the Industrial Farm Products Research Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, through the courtesy of Dr. G. E. Ward, may be dissolved in hot water to form an approximately 10 per cent solution, the zinc removed with $H_2S$, the precipitate washed thoroughly with hot water, and the lactic acid extracted with ether after moderate concentration in vacuum. The ether extraction is only necessary in order to insure minimum contamination with zinc, etc.

careful investigation of the effects, not only of iron, but of other elements. This has been carried out simultaneously with the experiments described above, and has proved to be exceedingly troublesome.

It was possible at any stage to show the necessity for Fe, when the Wadsworth and Wheeler (1934) technic of precipitating calcium phosphate in the medium, (shown by Pappenheimer and Johnson to remove excess Fe) was carried out. Iron salts alone were usually incapable of re-activating such treated media to the point where as good growth could be obtained as was given before the calcium treatment. The addition of hay ash, or the ash of other organic materials, in suitable concentration, almost always improved the growth when optimal, but not excessive, quantities of Fe were added, (as $FeCl_2 \cdot 6H_2O$ or $FeSO_4 \cdot 7H_2O$).

Numerous separations of the constituents of such ash materials were carried out by the usual methods of qualitative inorganic analysis, selecting procedures which were applicable in the presence of Ca and $PO_4$. The various fractions so obtained were tested for their effect on growth. Clear-cut results were difficult to obtain, a fact which was explicable in several ways: (*a*) Traces of many other elements than Fe undoubtedly occurred in the other components of the medium. (*b*) While the calcium treatment could be shown to remove most of the iron, it did not follow that the same would be true for other substances, or even that quantities of the same order of magnitude were present or required. (*c*) It was uncertain how effectively the analytical methods separated the usual metallic ions in the presence of relatively large amounts of Mg, Ca, $PO_4$, K and Na. (*d*) The extent to which rarer elements might be involved could not be foreseen.

As an illustration of these difficulties it may be noted that spectroscopic examination of one of the ash materials examined, (prepared from the butyl alcohol residue of a fibrin hydrolysate, kindly supplied by Prof. W. C. Rose of the Department of Biological Chemistry, University of Illinois) showed the presence of the following elements:

| | | | |
|---|---|---|---|
| Ca | Mn | Sn | Sr |
| Al | K | Sb | Mo |
| Fe | Na | Ba | Ni |
| Cu | Pb | Ag | Zn |
| Mg | B | Li | Ga |
| Si | In | P | Cr |

(Analysis done by Mr. Rockwell Kent, III, of the Massachusetts Institute of Technology.)

No effort has been made to purify the various organic and inorganic materials used in the preparation of test media. It was felt that any attempt to remove all the possible traces of foreign elements to a point two or three orders of magnitude below the range in which Fe, for example, inhibits toxin formation would be foredoomed to failure. That glassware, itself, may supply significant amounts of inorganic material is clearly indicated in the work of Pappenheimer and Johnson already cited. It seemed wiser to work the matter out as thoroughly as possible with materials readily and generally obtainable, leaving further refinements for future work, should it be indicated.

The results of numerous experiments with various types of control indicate quite clearly that the elements iron, manganese, copper and zinc are invariably essential for maximal growth, and further increases have been caused frequently, but not reproducibly, by cobalt, nickel and arsenic. Iodine and boron, probably also essential to protoplasmic development in general, have no effect on the growth of the test strain of diphtheria, and both undoubtedly occur as impurities in the medium in adequate concentration. In view of the striking effect of Fe on toxin production, as well as on growth, the whole subject of mineral requirements must naturally be re-investigated in relation to both matters with a standard toxin-producing organism. It seems unwarranted to extend the experiments with the Allen strain, and it is proposed to discontinue the work with it at this point.

It is not without interest or perhaps significance that these same metals, Fe, Mn, Cu and Zn (also Co) are now recognized as essential to many types of plant growth (see Biochemical Re-

views 1935 and 1936) and that, in addition, the plant physiologists, faced with the same difficulties of the occurrence as impurities of minute amounts of many elements, make use of the so-called "A-Z" solutions which contain traces of twenty or more additional elements, and in that way frequently obtain better growth.

As a result of many experiments, the following quantities of the various metals appear to be suitable.

| | γ per cc. |
|---|---|
| $FeSO_4 \cdot 7H_2O$ | 5.0 (would be inhibitory to toxin formation |
| $MnSO_4 \cdot 4H_2O$ | 2.5 |
| $CuSO_4 \cdot 5H_2O$ | 5.0 |
| ZnO (dissolved in HCl) | 2.5 |

Strikingly clear-cut experiments showing the effect of these various elements are not always obtained. The treatment with calcium evidently removes Fe quite effectively so that the concentration of this element giving optimal growth can be determined with some accuracy. The same is not true of the other metals, and the amounts employed may be considerably in excess of the actual requirements. They are, however, well below concentrations inhibitory to growth.

The following experiment illustrates the effect of Fe:

The basic medium was made according to formula A (last section), omitting the Fe. In order to remove the Fe present as impurities, all the constituents of the medium except the $MgSO_4$, which would be precipitated, the pimelic and nicotinic acids and $\beta$-alanine, which might be adsorbed on the precipitate, and the ethyl alcohol (volatile), were mixed, diluted to about $\frac{2}{3}$ the final volume and brought to about pH 8.4. For each 10 cc. lot, 3 mgm. $CaCO_3$ (dissolved in HCl) were added, and the solution boiled a few minutes and filtered through ashless paper. To the cooled filtrate the same amount of $CaCl_2$ was added and the procedure repeated. After adding the $MgSO_4$, accessories and alcohol, the filtrate was diluted to the desired volume, distributed in tubes and autoclaved at 10 pounds for 10 minutes. When cool, the Ca, Mn, Cu and Zn salts were added sterilely, and, finally, sterile $FeSO_4 \cdot 7H_2O$ as indicated in the table.

The tubes were incubated at 34° for 80 hours. Nitrogen determinations on the washed bacterial growth are given at the right.

| | $\gamma$ | mgm. N |
|---|---|---|
| 1. Control without $FeSO_4 \cdot 7H_2O$ | | 0.58 |
| 2. Control plus $FeSO_4 \cdot 7H_2O$ | 0.5 | 1.22 |
| 3. Control plus $FeSO_4 \cdot 7H_2O$ | 1.0 | 3.44 |
| 4. Control plus $FeSO_4 \cdot 7H_2O$ | 2.5 | 4.01 |
| 5. Control plus $FeSO_4 \cdot 7H_2O$ | 5.0 | 6.09 |
| 6. Control plus $FeSO_4 \cdot 7H_2O$ | 10.0 | 8.26 |
| 7. Control plus $FeSO_4 \cdot 7H_2O$ | 25.0 | 9.23 |
| 8. Control plus $FeSO_4 \cdot 7H_2O$ | 50.0 | 9.75 |
| 9. Control plus $FeSO_4 \cdot 7H_2O$ | 100.0 | 9.60 |

It appears that the medium still contained the equivalent of approximately 0.25 $\gamma$ $FeSO_4 \cdot 7H_2O$ per 10 cc. lot. The two calcium phosphate precipitates (from a total of 30 lots) were shown to contain approximately 0.54 mgm. Fe in the first, and 0.02 mgm. Fe in the second. This is equivalent to a $FeSO_4 \cdot 7H_2O$ concentration of nearly 100 $\gamma$ per lot originally present as impurities.

The iron could not always be removed as completely as was the case in this experiment and individual experiments with the other metals were often irregular and unsatisfactory. The entire matter may therefore be summed up in a further experiment which shows the effect of omission from the completed media of each in turn of the metals under consideration. The compositions of formulae *A* and *B* are given in the final section of the paper, and the general procedure was identical with that described above, including two precipitations with calcium phosphate.

| | mgm. N |
|---|---|
| 1. Medium A complete | 8.15 |
| 2. Medium A without Fe | 3.82 |
| 3. Medium A without Mn | 7.88 |
| 4. Medium A without Cu | 5.40 |
| 5. Medium A without Zn | 6.01 |
| 6. Medium B complete | 7.04 |
| 7. Medium B without Fe | 2.76 |
| 8. Medium B without Mn | 6.66 |
| 9. Medium B without Cu | 4.31 |
| 10. Medium B without Zn | 4.35 |

## GENERAL SUMMARY

In order to bring together all the facts discussed above, as well as to summarize and compare the part played by each component of the medium, two further experiments may be given. In the first, a casein hydrolysate medium (*A*) was used, while pure amino acids replaced this material in the second (*B*). The composition of the two complete media may be seen from the protocols themselves. The first tube in each experiment is complete, while from each successive tube, a single material is omitted; all others being present in the quantities shown, in a volume of 10 cc. The $CaCl_2$ treatment was not used in this case, since it would have been too cumbersome, and the results indicate the presence of Fe, Mn and Zn present as impurities.

*Formula A*

| | gram | mgm. N |
|---|---|---|
| 1. Complete medium | | 8.90 |
| 2. Complete medium without casein hydrolysate | 0.1 | 0.49 |
| 3. Complete medium without aspartic acid | 0.025 | 8.32 |
| 4. Complete medium without glutamic acid hydrochloride | 0.025 | 8.50 |
| 5. Complete medium without cystine | 0.006 | 0.84 |
| 6. Complete medium without tryptophane | 0.0001 | 8.92 |
| 7. Complete medium without KCl | 0.004 | 5.22 |
| 8. Complete medium without $Na_2HPO_4$ | 0.3 | 4.62 |
| 9. Complete medium without $MgSO_4 \cdot 7H_2O$ | 0.01 | 1.81 |
| 10. Complete medium without pimelic acid | 0.000,001,5 | 5.30 |
| 11. Complete medium without nicotinic acid | 0.000,023 | 0.20 |
| 12. Complete medium without $\beta$-alanine | 0.000,023 | 0.21 |
| 13. Complete medium without ethyl alcohol | 0.07 (cc.) | 6.85 |
| 14. Complete medium without *d*-lactic acid | 0.175 | 2.44 |
| 15. Complete medium without $CaCO_3$ (in HCl) | 0.002 | 7.92 |
| 16. Complete medium without $FeSO_4 \cdot 7H_2O$ | 0.000,05 | 8.21 |
| 17. Complete medium without $MnCl_2 \cdot 4H_2O$ | 0.000,025 | 8.00 |
| 18. Complete medium without $CuSO_4 \cdot 5H_2O$ | 0.000,05 | 5.80 |
| 19. Complete medium without ZnO (in HCl) | 0.000,025 | 9.12 |

In addition, an amount of NaCl varying between perhaps 0.2 and 0.4 gram per lot is present, resulting from the neutralization of HCl in the various solutions by NaOH.

*Formula B*

| | gram | mgm. N |
|---|---|---|
| 1. Complete medium | | 7.68 |
| 2. Complete medium without *l*-cystine | 0.007 | 0.26 |
| 3. Complete medium without *dl*-valine | 0.02 | 6.90 |

| | gram | mgm. N |
|---|---|---|
| 4. Complete medium without *l*-methionine | 0.006 | 0.67 |
| 5. Complete medium without *l*-tyrosine | 0.005 | 7.84 |
| 6. Complete medium without *l*-proline | 0.0075 | 7.04 |
| 7. Complete medium without *l*-aspartic acid | 0.05 | 4.27 |
| 8. Complete medium without *d*-glutamic acid hydrochloride | 0.075 | 4.94 |
| 9. Complete medium without KCl | 0.004 | 1.97 |
| 10. Complete medium without $Na_2HPO_4$ | 0.3 | 0.06 |
| 11. Complete medium without $MgSO_4 \cdot 7H_2O$ | 0.01 | 0.26 |
| 12. Complete medium without pimelic acid | 0.000,001,5 | 6.22 |
| 13. Complete medium without nicotinic acid | 0.000,023 | 0.10 |
| 14. Complete medium without $\beta$-alanine | 0.000,023 | 0.13 |
| 15. Complete medium without ethyl alcohol | 0.07 (cc.) | 6.34 |
| 16. Complete medium without *d*-lactic acid | 0.175 | 0.39 |
| 17. Complete medium without $CaCO_3$ (in HCl) | 0.002 | 4.14 |
| 18. Complete medium without $FeSO_4 \cdot 7H_2O$ | 0.000,05 | 7.24 |
| 19. Complete medium without $MnCl_2 \cdot 4H_2O$ | 0.000,025 | 7.49 |
| 20. Complete medium without $CuSO_4 \cdot 5H_2O$ | 0.000,05 | 5.73 |
| 21. Complete medium without ZnO (in HCl) | 0.000,025 | 7.40 |

It may be readily observed to what extent the complete media are dependent upon each of their individual components. The presence of casein hydrolysate in formula *A* naturally obscures the picture in the case of certain of the items, such as cystine, aspartic and glutamic acids, K, $PO_4$, Mg, and Ca, because of the presence of larger or smaller quantities as impurities in the commercial casein.

The amino acid formula (*B*) does not produce quite as heavy growth as does the casein hydrolysate formula. It has not seemed worth while to spend more time in attempts to improve it, because of the variation in amino acid requirements between strains and the fact that the Allen strain is not to be further investigated at this time. It is evident that neither valine, tyrosine nor proline, individually, exert any very marked effect. In numerous other experiments the general trend of results has indicated a distinct advantage when all are present. Moreover, the addition of several additional amino acids, together, usually gives somewhat better growth. Probably at this level the less that is demanded in the way of gross synthetic processes the better the growth that will result. To what extent these results can be applied to the production of toxin, and wherein lie the differences in toxin-producing ability of different strains of the

diphtheria bacillus, would seem to be problems which can now be approached with the aid of reproducible media.

## CONCLUSION

Growth of a strain of diphtheria bacillus, previously giving approximately 2.25 bacterial nitrogen on 10 cc. of a medium of simple and largely known composition can be increased three to four fold by increasing the proportions of certain of the components, notably cystine and *d*-lactic acid, and by adding traces of Fe, Mn, Cu and Zn.

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# FLAGELLA STAINING AS A ROUTINE TEST FOR BACTERIA[1]

H. J. CONN AND GLADYS E. WOLFE

*New York State Agricultural Experiment Station, Geneva, N. Y.*

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Nearly all descriptions of bacterial species contain statements as to whether or not motility is observed. The unsatisfactory character of this test has been realized for some time and various efforts have been made to improve upon the usual method, i.e., the microscopic examination of a hanging-drop. The most notable attempt of this sort in recent years is that by Tittsler and Sandholzer (1936), which is a macroscopic test depending upon the type of growth produced in a semi-solid agar.

In employing this semi-solid agar test on a series of soil organisms, certain plant pathogens, and organisms of the *Alcaligenes fecalis* and violet chromogenic groups, it was soon observed that, just as claimed by its originators, the test picks out many organisms that would not be recognized as motile by examination under the microscope. It has two disadvantages, however: it is rather slow, as cultures must be kept 3 or 4 days before a satisfactory reading can be made; it is uncertain, for considerable experience is necessary before it can be properly interpreted, and even an experienced user of the technic finds many tubes which give indefinite results. There proved, moreover, to be numerous cultures which appeared immotile in hanging-drop, and gave somewhat indefinite results by the semi-solid agar test, but which were shown on staining to have unmistakable flagella.

This raised the question as to whether it might be possible to devise a flagella-staining technic simple enough to be employed in routine pure culture study in the place of the conventional

[1] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 270.

test for motility. This has never been tried in the past because flagella staining methods are notoriously complicated and often fail to give identical results in the hands of different workers. Very recently, however, Hofer and Wilson (1938) have proposed a modification of the Gray flagella stain especially adapted for application to slime-forming bacteria. The results obtained with this technic were so consistent and were obtained with so little trouble, even in the hands of inexperienced technicians, that it seemed hopeful to modify the technic slightly so as to apply to other than slime-forming organisms. It was found easily possible to do this, the principal modification being the length of time to allow the organism to stand in water before making the smear on the slide. The procedure now employed in the case of miscellaneous bacteria is as follows:

### PREPARATION OF GLASS SLIDES

Use new slides if possible. (This is because under the drastic method of cleaning to remove grease, old slides have a greater tendency to break, due to crystallization of the glass.) Clean first in a dichromate cleaning fluid, wash in water and rinse in 95 per cent alcohol; then wipe with a clean piece of cheese cloth. (Wiping is not always necessary but is advisable unless fresh alcohol is used after every few slides.) Pass each slide back and forth through a flame for some time, ordinarily until the appearance of an orange color in the flame; some experience is necessary before the proper amount of heating can be accurately judged.

Cool slides gradually in order to re-anneal, and thus to minimize breakage. A crude but ordinarily satisfactory method of annealing is to place the flamed slides on a metal plate (flamed side up) standing on a vessel of boiling water; and then to remove the flame under the water so as to allow gradual cooling. (Failure to anneal may result in breakage, sometimes as long as two weeks after the heating.)

### PREPARATION OF SUSPENSIONS

Use young and actively growing cultures (e.g., 18–22 hours old), on agar slants. With a flamed *but well-cooled* loop transfer

a small amount of growth to 5–10 cc. of sterile distilled water, which has been held for several hours at room temperature. (Poor slides result from suspensions made in water that is too hot or too cold.)

Mix thoroughly in the distilled water and allow to stand 5–30 minutes, according to the type of growth produced. Gum-forming bacteria require 30 minutes, as recommended by Hofer and Wilson; those that produce no gum must stand in the water only 5–10 minutes. Standing in the water should be just long enough to allow the flagella to become untangled; too long a time results in their breaking off.

With a loop that has been flamed *and cooled*, remove a drop from the top of the suspension and place it on a glass slide prepared as above described. (The reason for taking material from near the surface of the suspension is because the non-motile cells tend to settle, while the motile cells—at least in the case of aerobes—collect at the top.) Smear the drop over the slide with the use of a second slide, as in preparing blood films; the film should be thin enough to dry rapidly and thus to minimize distortion.

## STAINING

Use the mordant recommended by Gray (1926): 5 cc. saturated aqueous solution potassium alum; 2 cc. saturated aqueous solution mercuric chloride; 2 cc. 20 per cent aqueous solution tannic acid; 0.4 cc. saturated alcoholic solution basic fuchsin (presumably about 6 per cent, which was the strength employed in the present work). For best results, filter just before using. The technic is essentially that of Gray. Apply cold for 8–10 minutes; 10 minutes is ordinarily best, but this varies with the organism studied. (More than 10 minutes of mordanting is apt to cause too much precipitate.)

After mordanting, wash slides about 10 seconds in running water. Dry in the air, without heating. Stain 5 minutes, without heating, with Ziehl's carbol fuchsin; wash in running water; dry and examine. (In the present work a recently certified batch of National Aniline basic fuchsin was employed.)

In this procedure the most crucial points are the proper amount of heat to give the slides and the method of cooling so as to minimize the tendency to break. Although not all breakage can be avoided, the above-described method of cleaning the slides is recommended because of the better demonstration of flagella than when the preparations are made on slides cleaned by more ordinary procedures.

The technic thus developed proves simple enough so that it seems possible to recommend it as a routine test. It takes longer, to be sure, than the conventional hanging-drop test; but it gives a much higher percentage of positives. Results can be obtained much more quickly than by the semi-solid agar test of Tittsler and Sandholzer; they are more reliable, because interpretation of the results is never open to question; and finally, information is thus secured not only in regard to motility but also as to the type of flagellation. It is, in fact, hardly more difficult than the Gram stain, giving results that are more clear-cut and more easily interpreted.

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# THE PROTEOLYTIC ENZYMES OF BACTERIA

## II. The Peptidases of Some Common Bacteria[1]

JULIUS BERGER, MARVIN J. JOHNSON, AND W. H. PETERSON

*Departments of Biochemistry and Agricultural Bacteriology, University of Wisconsin, Madison*

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### INTRODUCTION

A great deal of work has been done on the proteolytic enzymes of bacteria, especially those enzymes which are secreted into the medium by the living cell or as the result of its autolysis. The proteolytic enzymes of the cells themselves, however, have been less studied. Studies on bacterial peptidases in which substrates of known formulae (such as synthetic peptides) have been used, are few and fragmentary. In most cases, only a few data illustrating the fact that the peptides were hydrolyzed are given. A brief literature review is given in table 1.

In view of this lack of information, it was decided to investigate how the peptidase systems of bacteria of widely differing genera compared with one another and with the systems found in molds (Johnson and Peterson, 1935; Berger, Johnson and Peterson, 1937), yeast (Grassmann, 1930; Macrae 1933), and animal erepsin (Johnson, Johnson and Peterson, 1936; Johnson 1937). The first paper of this series (Berger, Johnson and Peterson, 1938) described in some detail the properties of the peptidases of *Leuconostoc mesenteroides*. The present paper will give a survey of the peptidases found in twelve organisms which were chosen so as to represent aerobic, anaerobic, facultative,

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proteolytic and non-proteolytic bacteria. The organisms were obtained from the stock cultures of the Bacteriology Department[2] and were as follows: *Escherichia coli*, *Bacillus megatherium*,

TABLE 1

*Literature review of bacterial peptidases*

| ORGANISM | SUBSTRATE HYDROLYZED | pH OPTIMUM | AUTHOR |
|---|---|---|---|
| *Staph. pyogenes-aureus* | Glycyl-*l*-tyrosine | (7)* | Otsuka, 1916 |
| *B. prodigiosus* | Glycyl-*l*-tryptophane | | |
| *B. pyocyaneus*† | Leucylglycine | 8.4 | Gorbach, 1930 |
| | Leucyldiglycine | 8.4 | |
| *B. casei* | Leucylglycine | (7.5) | Tarnanen, 1930 |
| | Glycylalanine | (7.5) | |
| *Enterococcus, Gorini* | Leucylglycine | (7.8) | Gorini, Grassmann, Schleich, 1932 |
| | Leucyldiglycine | (7.0) | |
| *Mammococcus, Gorini* | Leucylglycine | 4.8 | Gorbach and Ulm, 1935 |
| *B. fluorescens* | Diglycine | 8.4 | Gorbach and Pirch, 1936 |
| | Leucylglycine | 8.4 | |
| | Leucyldiglycine | 8.4 | |
| *Cl. histolyticum*† | Leucyldiglycine | (7.8) | Weil and Kocholaty, 1937 |
| *Caseicoccus, Gorini* | Leucylglycine | 4.8, 7.0 | Gorbach, 1937 |
| *Gastrococcus, Gorini* | Leucyldiglycine | 4.8 | |
| Many aerobes, anaerobes† | Leucylglycine | (7.8) | Maschmann, (6 papers 1937–38) |
| | Leucyldiglycine | (7.2) | |
| *Staph. aureus* | Benzoyldiglycine | 8.0 | Imaizumi 1938a, 1938b |
| *Staph. aureus, B. typhi* | Leucyldiglycine | 8.0 | |
| *Staph. aureus, B. typhi* and *B. subtilis* | Chloroacetylphenylalanine | 7.0 | |
| 14 organisms | Glycylphenylalanine | (8.0) | |
| 14 organisms | Diglycine | 8.0 | |
| 6 organisms | Benzoyldiglycine | (8.0) | |

* Figures in parentheses represent the pH values at which the determination was made, a pH optimum not having been determined.

† Culture filtrates were used as enzyme source; in case of other organisms cell extracts were used.

*Proteus vulgaris*, *Pseudomonas fluorescens*, *Bacillus mesentericus*, *Bacillus subtilis*, *Clostridium butylicum* 21, *Clostridium sporogenes*,

[2] The authors with to thank Dr. Elizabeth McCoy and Miss Elizabeth Krauskopf for supplying transfers of these organisms.

*Clostridium acetobutylicum* (Pike strain), *Lactobacillus pentosus* 124-2, *Propionibacterium pentosaceum* P-11 and *Phytomonas tumefaciens*.

## EXPERIMENTAL

### *Methods*

When this survey was undertaken, it was recognized that if any one procedure were adopted, it could not represent the optimum conditions for the study of all the organisms. The extent to which different organisms would vary in the nature of their peptidases was, of course, unknown, but, as a first approximation, it was assumed that their properties would fall between the limits of those of the peptidases of molds, yeast and erepsin. For example, hydrolyses were carried out at pH 8 since known peptidase systems act optimally close to this value. Since a certain degree of uniformity was desirable, it was decided to keep constant as far as practicable, the composition of the medium, the growth period of the organisms and the method of enzyme extraction. Because of these limitations, the data reported in this paper are applicable only for the conditions and methods by which they were obtained.

The organisms were grown in 20-liter Pyrex bottles, each containing 14 liters of 0.5 per cent glucose, 0.5 per cent Bacto-peptone medium made with tap water. The pH of this medium was 6.8. Six hundred cubic centimeters of a 24-hour culture grown in the same medium were used as inoculum. All cultures were incubated at 37° for 24 hours; the cells were collected by centrifuging with a Sharples supercentrifuge and then placed in the freezing compartment of a refrigerator. These wet cells usually contained 80 to 85 per cent water. In the case of the anaerobes, no special precautions were taken to preserve anaerobiosis since the depth of the medium in the Pyrex bottles was about 30 cm. In the case of the aerobes (the first six organisms listed above), the medium was aerated throughout the whole period of incubation by bubbling through it filtered, sterile air at a rate which was limited by the amount of frothing. Unfortunately, this varied with the different organisms. In all cases the cells were

examined microscopically for freedom from contamination before harvesting.

*Extraction of cells.* With *Bacillus megatherium*, a number of extraction methods were studied. The results of these experiments can be stated briefly as follows: Peptidases were extracted from fresh cells with difficulty (in keeping with the experience of many other investigators), but repeated freezing and thawing of the cells made the enzymes readily available for extraction by a number of procedures. These included autolysis in 40 per cent aqueous glycerol, autolysis under toluene of cells previously dried by acetone-ether treatment, and autolysis of wet cells with ethyl acetate and ether. Preliminary studies on the extraction of peptidases from *Escherichia coli* gave results very similar to those obtained above. On the basis of experiments with *Bacillus megatherium* and *Leuconostoc mesenteriodes* it was concluded that keeping bacteria in a frozen condition as long as three months did not result in a decrease in peptidase activity. It was therefore decided to use the following method in general for all the organisms:

Cells were frozen and thawed at least 5 times over a period of not less than a week, and then ground with sand in a mortar. Toluene was added and the pH was maintained at 6.8 to 7.0 by the careful addition of 0.1 N NaOH. The mass was then diluted to a volume which was roughly 5 to 10 times that of the cells and the suspension was allowed to extract at room temperature, usually for 24 hours. The solution was centrifuged in an angle head centrifuge at 4000 R.P.M. until it was clear. This crude enzyme solution was then analyzed for its ability to hydrolyze various substrates. In some cases, where the cells were poor in peptidases or the efficiency of the extraction was low, it was necessary to concentrate the crude enzyme solution *in vacuo* below 40°, in order to obtain a solution of sufficient activity to make determinations within reasonable lengths of time. This concentrate was then dialyzed against distilled water, usually for about 15 hours, to remove some of the impurities. The enzyme solutions were stored in a refrigerator at 2° to 5° in the

presence of toluene. Whenever an enzyme-substrate mixture was incubated for a long period where bacterial growth might occur, toluene was always added.

The extent of hydrolysis of the peptide substrates used was determined by the Linderstrøm-Lang titration (Linderstrøm-Lang, 1927). In all cases the substrate concentration was M/30 (M/15 for racemic peptides). Unless otherwise mentioned, the substrates were used at pH 7.8 to 8, being half neutralized with NaOH. They contained no added buffers. For all determinations of pH optima for hydrolysis of peptides, the regular substrates were adjusted to the desired pH by the addition of predetermined amounts of 1 N acetic acid or 1 N NaOH solution. Thus, there was no buffering other than that provided by the peptides themselves in the neighborhood of pH 8 and by the small amount of acetate at the lower pH values. The pH values of the enzyme-substrate mixtures were determined after the incubation period by means of the glass electrode. One cubic centimeter samples were titrated from 3.0 cc. of reaction mixture, made by diluting 2.0 cc. of peptide solution to a total of 3.0 cc. with enzyme and water. The maximum variation between duplicate determinations was equivalent to 2 to 5 per cent hydrolysis of the substrate, depending upon the amount of dissolved materials in the enzyme solution, since these sometimes interfered with the accurate reading of the end-point. In all cases, hydrolysis values have been reported as per cent hydrolysis of one optical component of racemic peptides.

### *The peptidases of aerobes*

*Proteus vulgaris.* The rate of aeration of the medium in the growth of this organism was very much limited by the excessive frothing which occurred. From 14 liters of medium, 5.1 grams wet cells were obtained. The enzyme solution prepared from these cells (10 hour autolysis) was concentrated *in vacuo* and dialyzed against distilled water for 16 hours. The analysis of the final solution is given in table 2.

Johnson, Johnson and Peterson (1936) found that leucyl-

peptidase of hog erepsin was activated by Mg ions and that it hydrolyzed LG[3] and LGG at equal rates. Tests for $Mg^{++}$ activation were therefore made with several bacterial enzyme solutions to see if any of these contained enzymes resembling animal leucylpeptidase. The solutions before testing were reduced in Mg content by acetone precipitation. The data of

TABLE 2

*Peptidases of Proteus vulgaris*

| SUBSTRATE | CRUDE ENZYME* | | | HYDROLYSIS BY ACETONE-PRECIPITATED ENZYME† | | RECOVERY OF ENZYME AFTER PRECIPITATION |
|---|---|---|---|---|---|---|
| | Time of incubation | Hydrolysis‡ | Yield per 10 grams wet cells | No Mg | 0.003 M Mg | |
| | *hours* | *per cent* | *cc.§* | *per cent* | *per cent* | *per cent* |
| *dl*-AG | 1 | 20 | 13 | 25 | 27 | 6 |
| *dl*-LG | 3 | 20 | 4.3 | 14 | 76 | 48 |
| GG | 13 | 34 | 1.6 | 8 | 5 | 8 |
| *dl*-AGG | 15 | 29 | 1.3 | 6 | 7 | 13 |
| *dl*-LGG | 3 | 23 | 4.9 | 18 | 73 | 40 |
| GGG | 15 | 8 | 0.3 | 2 | 2 | 16 |

* 0.50 cc. (representing 77 mgm. of wet cells) of crude enzyme was used per determination.

† For demonstration of $Mg^{++}$ activation, 8.0 cc. of crude solution at pH 6.3 were mixed with 16 cc. acetone, let stand 1 minute, and centrifuged. The precipitate was suspended in 8 cc. water, and recentrifuged. 0.50 cc. of the resulting clear solution was used in all cases; incubation was for 24 hours.

‡ Per cent hydrolysis of one optical component of racemic peptides. 0.003 M $MgCl_2$ was present in the substrates.

§ The basis for calculating yields has been described in a previous paper (Berger, Johnson and Peterson (1938)). Briefly stated, the values represent the hydrolysis which would be obtained by an extract from 10 grams wet cells in one hour at 40°, expressed as cc. of 0.2 N HCl.

table 2 show that the hydrolysis of LG and LGG by the leucylpeptidase of *Proteus vulgaris* was activated by $Mg^{++}$. Furthermore, the two peptides were hydrolyzed at approximately the same rate by the crude enzyme solution as well as by the

[3] In this paper the following abbreviations will be used: LG = leucylglycine, LGG = leucyldiglycine, AG = alanylglycine, AGG = alanyldiglycine, GG = diglycine, GGG = triglycine.

acetone-precipitated preparation, both in the absence and presence of Mg ions.

*Pseudomonas fluorescens.* 6.8 grams wet cells were obtained from 14 liters of medium. Here too, the rate of aeration was greatly reduced on account of excessive frothing of the medium. The enzyme solution prepared from these cells (10 hour autolysis) was concentrated *in vacuo* and dialyzed 16 hours. The analysis is given in table 3. It may be seen that the hydrolysis of LG,

TABLE 3

*Peptidases of Pseudomonas fluorescens*

| SUBSTRATE | CRUDE ENZYME* | | | HYDROLYSIS BY ACETONE-PRECIPITATED ENZYME† | | | RECOVERY OF ENZYME AFTER PRECIPITATION |
|---|---|---|---|---|---|---|---|
| | Time of incubation | Hydrolysis | Yield per 10 grams wet cells | Time of incubation | No Mg | 0.003 M Mg | |
| | *hours* | *per cent* | *cc.* | *hours* | *per cent* | *per cent* | *per cent* |
| *dl*-AG | 2 | 36 | 8.7 | 2 | 20 | 20 | 56 |
| *dl*-LG | 2 | 62 | 15 | 8 | 14 | 57 | 23 |
| GG | 15 | 52 | 1.2 | 9 | 28 | 31 | 100 |
| *dl*-AGG | 2 | 30 | 7.3 | 8 | 36 | 41 | 34 |
| *dl*-LGG | 2 | 56 | 13 | 8 | 26 | 50 | 22 |
| GGG | 15 | 78 | 2.9 | 8 | 26 | 40 | 96 |

* 0.50 cc. (corresponding to 103 mgm. wet cells) of crude enzyme solution was used per determination. Substrates contained 0.003 M $MgCl_2$.

† For demonstration of $Mg^{++}$ activation, 8 cc. crude solution were mixed with 16 cc. acetone and centrifuged after 3 minutes. The resulting precipitate was suspended in 8 cc. $H_2O$, recentrifuged and 0.50 cc. of clear solution used per determination.

Basis for calculating yields same as in table 2.

LGG, GGG and possibly AGG was activated by $Mg^{++}$. It is very interesting to note that the GG- and GGG-splitting enzymes were recovered completely after acetone precipitation, whereas the enzymes of animal erepsin and mold which split these peptides are almost completely destroyed by acetone precipitation at room temperature.

*Bacillus subtilis.* 15.1 grams wet cells from 14 liters of medium were frozen 6 weeks with intermittent thawing, then autolyzed with toluene for 24 hours. The clear solution obtained after cen-

trifuging was tested for the presence of Mg-activated enzymes. As shown in table 4, the hydrolysis of LG was activated but the lack of activation of LGG hydrolysis was rather surprising since with *Proteus*, *Pseudomonas* and animal leucylpeptidase both LG and LGG hydrolyses were activated. The stability of the GGG-splitting enzymes to acetone precipitation was markedly different from the behavior of the GGG-splitting enzyme in erepsin towards this treatment and resembled that found in *Pseudomonas fluorescens* (table 3). LGG was found to be hydrolyzed by the

TABLE 4

*Peptidases of B. subtilis*

| SUBSTRATE | CRUDE ENZYME* | | HYDROLYSIS BY ACETONE-PRECIPITATED ENZYME† | | | RECOVERY OF ENZYME AFTER PRECIPITATION |
|---|---|---|---|---|---|---|
| | Time of incubation | Hydrolysis | Time of incubation | No Mg | 0.003 M Mg | |
| | *hours* | *per cent* | *hours* | *per cent* | *per cent* | *per cent* |
| *dl*-AG | 6 | 22 | | | | |
| *dl*-LG | 24 | 70 | 24 | 46 | 70 | 100 |
| GG | 24 | 12 | | | | |
| *dl*-AGG | 1 | 60 | 1 | 71 | 71 | 118 |
| *dl*-LGG | 1 | 60 | 1 | 59 | 59 | 100 |
| GGG | 24 | 70 | 24 | 61 | 49 | 87 |

* 1.0 cc. crude enzmye solution (corresponding to 125 mgm. wet cells) was used per determination. The substrates contained 0.003 M $MgCl_2$.

† To test for $Mg^{++}$ activation, 10 cc. crude enzyme solution at pH 6.84 were mixed with 10 cc. of acetone; the precipitate obtained was centrifuged after 3 minutes, then suspended in 10 cc. $H_2O$, and recentrifuged. 1.0 cc. of the clear solution was used per determination.

acetone-precipitated enzyme preparation at a pH optimum of 7.7 as shown in figure 1.

*Escherichia coli.* During the course of this work 100 grams of wet cells were grown aerobically. The average yield was 25 grams from 14 liters of medium (strongly aerated). It was found that with *E. coli* just as with *B. megatherium* the peptidases were not readily extracted from fresh cells by a 17 hour autolysis in 40 per cent glycerol at 37°. (Only 12 units of LG-splitting enzyme were obtained per 10 grams wet cells.) Table 5 gives

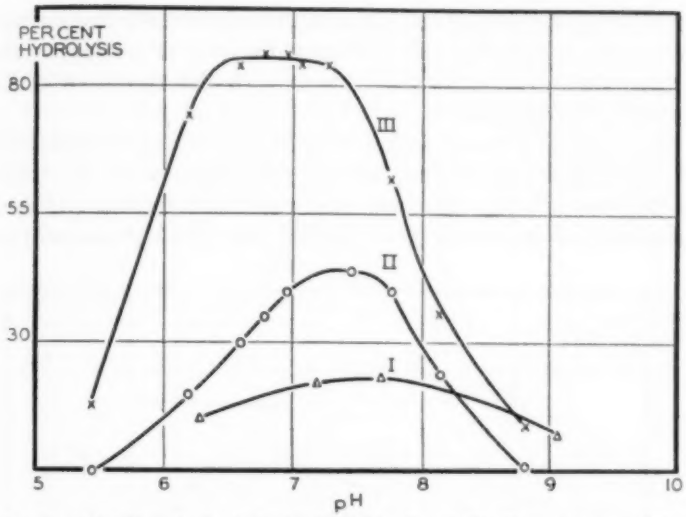


FIG. 1. pH optima for hydrolysis of LGG by *B. subtilis* (*I*, acetone-precipitated enzyme solution, incubation period 1 hour), and *C. sporogenes* (*II*, after 1 hour incubation, *III*, after 4 hours incubation at 40°).

TABLE 5

*Peptidases of E. coli*

| SUBSTRATE | AEROBIC* | | ANAEROBIC† | |
|---|---|---|---|---|
| | Hydrolysis | Yield per 10 grams wet cells | Hydrolysis | Yield per 10 grams wet cells |
| | *per cent* | *cc.* | *per cent* | *cc.* |
| *dl*-AG | 94 | 127 | 24 | 7.0 |
| *dl*-LG | 41 | 56 | 18 | 5.2 |
| GG | 50 | 68 | 16 | 4.7 |
| *dl*-AGG | 67 | 91 | 52 | 15 |
| *dl*-LGG | 34 | 46 | 68 | 39 |
| GGG | 40 | 54 | 11 | 2.9 |

* 24 grams of wet cells were frozen 2 days with intermittent thawing, then autolyzed for 4 hours. 0.4 cc. crude enzyme solution, corresponding to 36.9 mgm. wet cells, was used per determination. Incubation time was one hour.

† 1.0 cc. crude enzyme solution, corresponding to 171 mgm. wet cells, was used per determination. Incubation time was 1 hour. Substrates contained 0.003 M $MgCl_2$.

Basis for calculating yields same as in table 2.

the results of the analyses on a representative enzyme preparation. The yield of peptidases may be seen to be higher than for any organism yet studied by us with the exception of *Leuconostoc mesenteroides*.

Space will not be taken here to present the experimental data on the kinetics of the peptidases, since they were determined by the use of crude solutions. It was found that at pH 8, the hydrolysis of AG, LG, AGG, LGG and GGG proceeded at a

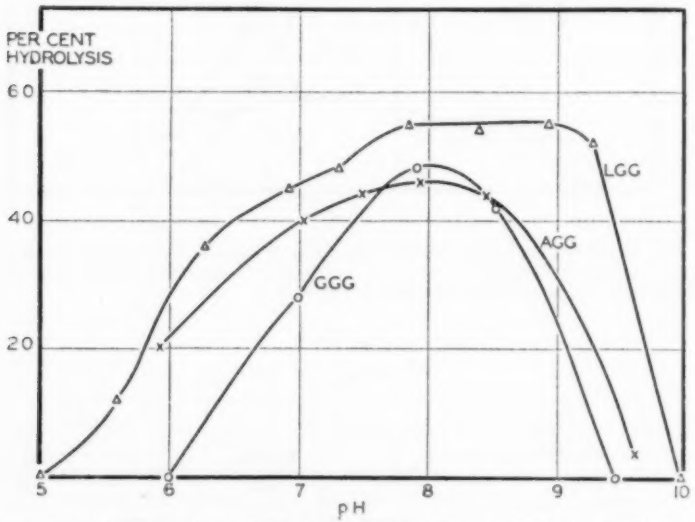


FIG. 2. pH optima for hydrolysis of LGG, AGG and GGG by peptidases of *E. coli*. Incubation time was 1 hour at 40°.

constant rate while the hydrolysis of GG was a first order reaction. That *E. coli* contains a leucylpeptidase was shown by the fact that in the presence of 0.003 M $Mg^{++}$, the hydrolysis of LGG by an acetone-precipitated enzyme preparation was activated fourfold, namely from 10 to 44 per cent.

Figures 2 and 3 summarize the data obtained for the optimum pH values for the hydrolysis of peptides by *E. coli* peptidases. From the shape of the curves obtained for LG and LGG hy-

drolysis, it appears that in both cases a second enzyme with pH optimum at 6 to 7 is involved. The very rapid decrease in the rate of hydrolysis of these two peptides after pH 9.3 and 9.6 is possibly due to the precipitation of the metal activator as $Mg(OH)_2$.

When *E. coli* was grown anaerobically, 8.6 grams wet cells (from 14 liters of medium) were obtained. The frozen cells were autolyzed 24 hours, and the resulting enzyme preparation, after

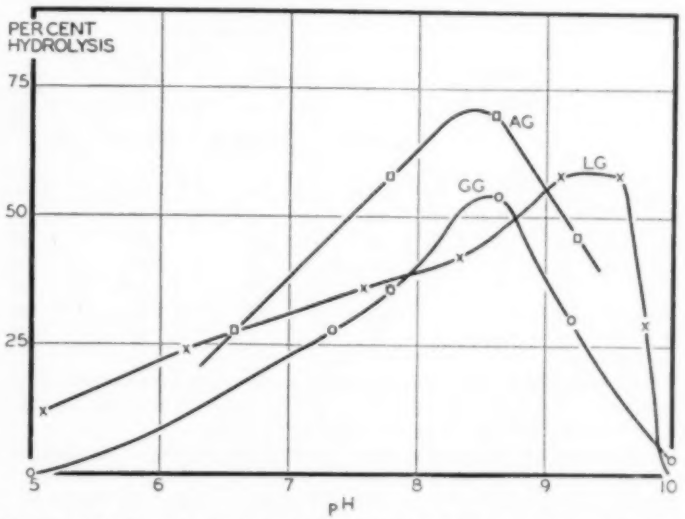


FIG. 3. pH optima for hydrolysis of AG, LG and GG by peptidases of *E. coli*. Incubation time was 1 hour at 40°.

clarification, was analyzed on 6 peptides. The results are given in table 5. When grown anaerobically, the cells appeared to contain less enzyme than when grown aerobically. Furthermore, the leucyl peptides were hydrolyzed more rapidly than the glycyl peptides, a reversal of the picture presented by the aerobically grown cells. A test for Mg-activatable peptidases gave negative results.

*Bacillus megatherium*. During the course of this study, 650

grams of wet cells were grown. The average yield was 40 grams wet cells per 14 liters of medium. Table 6 gives the data obtained for analyses on 6 peptides with a representative enzyme preparation. When a crude enzyme solution was used, it was found that the hydrolyses of LG, AG, GG and LGG were first order reactions while GGG and AGG were linear.

TABLE 6

*Peptidases of B. megatherium*

| SUBSTRATE | ENZYME SOLUTION PER DETERMINATION | PREPARATION VIII* | | | |
|---|---|---|---|---|---|
| | | Dialyzed enzyme solution† | | Acetone-precipitated enzyme‡ | Recovery of enzyme after precipitation |
| | | Hydrolysis | Yield per 10 grams wet cells | Hydrolysis | |
| | *cc.* | *per cent* | *cc.* | *per cent* | *per cent* |
| *dl*-AG | 0.1 | 68 | 71 | 30 | 44 |
| *dl*-LG | 0.3 | 48 | 17 | 20 | 42 |
| GG | 0.3 | 40 | 14 | 14 | 35 |
| *dl*-AGG | 0.3 | 54 | 19 | 14 | 26 |
| *dl*-LGG | 0.1 | 46 | 48 | 24 | 52 |
| GGG | 1.0 | 14 | 1.5 | 7 | 25 |

* Preparation VIII—62 grams wet cells were frozen and thawed 6 times during 2 days, then autolyzed with toluene for 12 hours. After clarification, the solution was concentrated in vacuo and dialyzed 10 hours.

† 1 cc. dialyzed solution corresponds to 480 mgm. wet cells. Incubation time was 1 hour.

‡ Acetone-precipitation—5 cc. dialyzed enzyme concentrate (pH 6.55) were mixed with 10 cc. acetone. The resulting precipitate was immediately centrifuged off, then suspended in 10 cc. $H_2O$. All substrates were incubated 2 hours, except GGG (4 hours).

Basis for calculating yields same as in table 2.

Acetone, ethyl alcohol, methyl alcohol, dioxan and combinations of some of these were used as precipitating agents. On the average, 40 to 50 per cent of the enzyme was recovered with acetone (table 6) or dioxan but less than 10 per cent with ethyl or methyl alcohol. When acetone was used to precipitate from a dilute enzyme solution, practically complete recovery of LG-, GG- and LGG-splitting enzymes was obtained. Preliminary

experiments on adsorption of enzymes on $\beta$-$Fe_2O_3 \cdot H_2O$ in aqueous solution showed that at pH 6.04, from dilute solution, the LGG-splitting enzyme was preferentially adsorbed, leaving behind in the filtrate a purer LG- and GG-splitting solution. $Al(OH)_3$-$C_\gamma$ also adsorbed the LGG-splitting enzyme preferentially but did not give as sharp a separation.

A number of times enzyme preparations of *B. megatherium* were acetone-precipitated and the resulting preparations tested

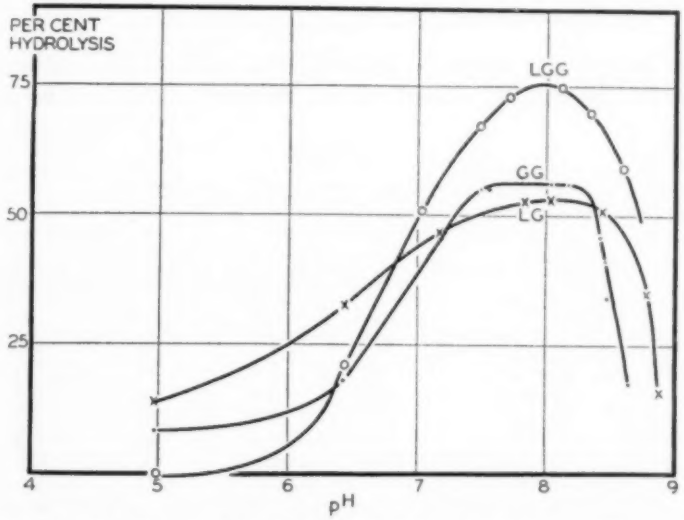


FIG. 4. pH optima for hydrolysis of LG, GG and LGG by peptidases of *B. megatherium*. Incubation time was 1 hour.

for the hydrolysis of the usual 6 peptides in the presence and absence of 0.003 M $Mg^{++}$. No activation was observed at any time. Another procedure to test for $Mg^{++}$ activation was also tried. An enzyme solution was dialyzed until it had decreased 50 per cent in activity and then was tested on LG and LGG in the presence and absence of $Mg^{++}$. No activation was observed.

From figure 4 it may be seen that the pH optimum for hydrolysis of GG was 7.6 to 8.0 and of LG and LGG, 8.0.

*Phytomonas tumefaciens.* This organism was grown on a synthetic medium of the following composition:[4]

| | |
|---|---|
| Sucrose | 10.0 grams |
| $(NH_4)_2SO_4$ | 7.5 grams |
| Glutamic acid | 2.5 grams |
| $MgSO_4 \cdot 7H_2O$ | 0.2 gram |
| NaCl | 0.2 gram |
| $K_2HPO_4$ | 10.0 grams |
| $CaCl_2$ | 0.1 gram |
| Distilled water | 1000 cc. |

pH was adjusted to 6.8

The culture was grown in 6-liter Pyrex bottles with continuous aeration by sterile, filtered air for 88 hours at 26°. The cells

TABLE 7

*Peptidases of P. tumefaciens and B. mesentericus*

| SUBSTRATE | P. TUMEFACIENS* | | B. MESENTERICUS† | |
|---|---|---|---|---|
| | Hydrolysis | Yield per 10 grams wet cells | Hydrolysis | Yield per 10 grams wet cells |
| | *per cent* | *cc.* | *per cent* | *cc.* |
| *dl*-AG | 64 | 3.1 | 14 | 1.9 |
| *dl*-LG | 78 | 3.7 | 10 | 1.4 |
| GG | 50 | 2.4 | 2 | 0.3 |
| *dl*-AGG | 26 | 1.2 | 34 | 4.7 |
| *dl*-LGG | 48 | 2.3 | 42 | 5.8 |
| GGG | 10 | 0.5 | 6 | 0.8 |

* 1.0 cc. crude enzyme solution corresponding to 210 mgm. wet cells was used per determination. Incubation time was 5 hours.

† 1.0 cc. dialyzed enzyme solution, corresponding to 180 mgm. wet cells was used per determination. Incubation time was 2 hours.

Basis for calculating yields same as in table 2.

were centrifuged off, frozen two weeks and thawed intermittently at least 5 times. They were then suspended in water, the pH adjusted to 7 and the mixture allowed to autolyze with toluene at room temperature. Samples were analyzed daily for LG- and LGG-splitting enzymes until no further increase appeared

[4] This medium was devised by J. M. Van Lanen, A. J. Riker and I. L. Baldwin at the University of Wisconsin. It gives a yield of 70 grams wet cells (considerable of which appears to be gum) from 14 liters of medium.

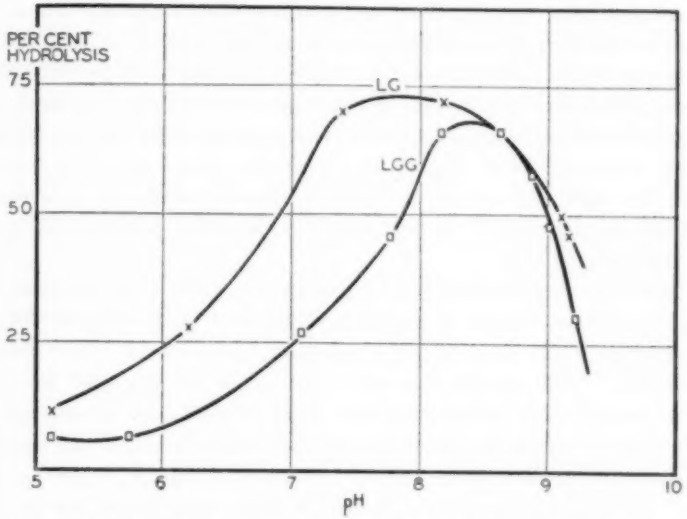


FIG. 5. pH optima for hydrolysis of LG and LGG by peptidases of *P. tumefaciens*. Incubation time was 6 hours at 40°.

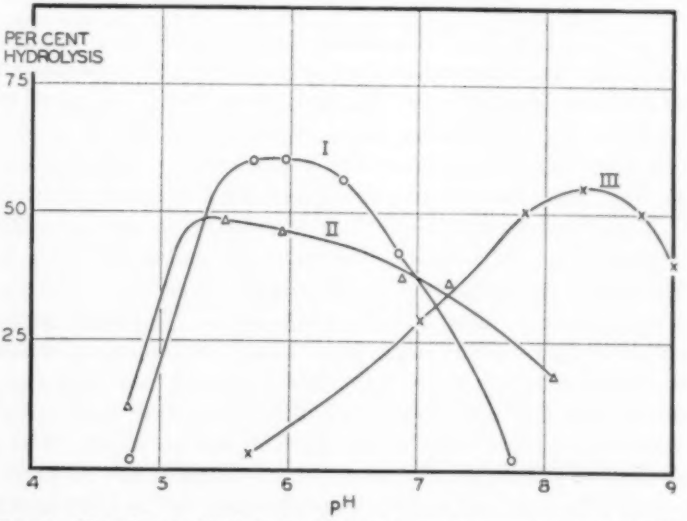


FIG. 6. pH optima for hydrolysis of AG by *P. pentosaceum* (*I*), and of LGG by *L. pentosus* (*II*) and *B. mesentericus* (*III*).

to occur (nine days). The solution was then filtered. Its analysis is summarized in table 7 (which includes also data for *Bacillus mesentericus*). *P. tumefaciens* also contains a Mg- activated leucylpeptidase since the hydrolysis of LGG by an acetone-precipitated enzyme preparation increased from 26 to 44 per cent when 0.003 M $Mg^{++}$ was added. From figure 5 it may be seen that the crude enzyme solution hydrolyzed LG at an optimum pH of 7.6 to 8.2, while LGG was hydrolyzed most rapidly at pH 8.4.

*Bacillus mesentericus.* 10.6 grams wet cells from 14 liters of medium were frozen 3 months, autolyzed with toluene for 24 hours, and the final enzyme solution dialyzed 14 hours before analysis. The results are given in table 7. It may be seen that leucyl and alanyl peptides were hydrolyzed more rapidly than glycyl peptides, a characteristic common to all the aerobic organisms studied with the exception of *Escherichia coli.* The pH optimum for the hydrolysis of LGG was found to be 8.4, as shown in figure 6.

### *Specificity of bacterial peptidases*

In table 8 are given the data for the hydrolysis of a number of peptides and their derivatives by peptidases of *Escherichia coli* and *Bacillus megatherium.* It may be seen that *E. coli* peptidases hydrolyze glycyl peptides more rapidly than leucyl peptides, while the reverse is true for *B. megatherium.* Substitution of a methyl group for a hydrogen atom of the free amino group such as in sarcosylglycine, N-methylalanyldiglycine and N-methylleucyldiglycine inhibited hydrolysis very markedly with both organisms. *B. megatherium* peptidases, however, hydrolyzed sarcosyldiglycine more rapidly than GGG. Acylated peptides were not split by either organism. For *B. megatherium,* separate determinations (not given in the table) showed that chloroacetylglycine was not hydrolyzed, while prolylglycine and prolyldiglycine were both very slowly hydrolyzed at about 1/50 the rate of LG. Prolylglycine was split 1.7 times as fast as prolyldiglycine. Decarboxylation of peptides such as in leucylmethyl-

amine and glycylmethylamine inhibited hydrolysis by peptidases of both organisms, but *E. coli* peptidases split both compounds very slowly. Yeast dipeptidase and presumably ereptic dipeptidase require a free carboxyl group on the dipeptide to be hydrolyzed (Bergmann *et al.*, 1935), but leucylpeptidase (Johnson

TABLE 8

*Hydrolysis of synthetic peptides by E. coli and B. megatherium peptidases*

Data are expressed as per cent hydrolysis of one optical component of racemic peptides. Thus 200 per cent splitting of AGG means that 2 linkages (presumably of the natural form of the peptide) have been completely hydrolyzed with the formation of 3 amino acid molecules.

| SUBSTRATE | E. COLI (AEROBIC PREP. II)* | | | B. MEGATHERIUM (PREP. VII)† | | |
|---|---|---|---|---|---|---|
| | Per cent hydrolysis of one linkage | | | | | |
| | 1 hour | 6 hours | 24 hours | 1 hour | 6 hours | 24 hours |
| *dl*-AG | 94 | 100 | 107 | 41 | 88 | 94 |
| *dl*-LG | 41 | 100 | 101 | 13 | 47 | 83 |
| *dl*-Leucylmethylamine | 4 | 9 | 12 | 1 | 5 | 5 |
| GG | 50 | 84 | 90 | 9 | 31 | 49 |
| Glycylmethylamine | 2 | 4 | 12 | 0 | 0 | 0 |
| Sarcosylglycine | 0 | 5 | 23 | 0 | 0 | 3 |
| Sarcosyl-*l*-tyrosine | 0 | 2 | 4 | 0 | 1 | 10 |
| *dl*-AGG | 67 | 184 | 201 | 11 | 47 | 138 |
| *dl*-N-Methylalanyldiglycine | 4 | 20 | 58 | 4 | 5 | 65 |
| *dl*-LGG | 34 | 162 | 202 | 36 | 102 | 136 |
| *dl*-N-Methylleucyldiglycine | 5 | 13 | 83 | 1 | 1 | 7 |
| GGG | 40 | 140 | 183 | 0 | 1 | 18 |
| Sarcosyldiglycine | 6 | 29 | 169 | 1 | 3 | 35 |
| Benzoyldiglycine | 0 | 1 | 1 | 0 | 0 | 0 |
| Tetraglycine | 33 | 164 | 272 | 2 | 4 | 16 |
| Chloroacetyl-*l*-tyrosine | 0 | 0 | 1 | 1 | 1 | 6 |

* 0.40 cc. crude enzyme solution (corresponding to 36.9 mgm. wet cells) was used per 3 cc. of reaction mixture in all determinations.

† 0.30 crude enzyme solution (corresponding to 50 mgm. wet cells) was used per 3 cc. of reaction mixture in all determinations.

*et al.*, 1936) is able to split decarboxylated dipeptides. Since *E. coli* contains an LGG-splitting enzyme whose activity is accelerated by $Mg^{++}$, and since decarboxylated dipeptides are hydrolyzed by the crude enzyme solution, the presence of an animal-leucylpeptidase-like enzyme is strongly suggested.

An activation appears to occur in the hydrolysis of N-methylleucyldiglycine and sarcosyldiglycine between 6 and 24 hours of incubation (*E. coli*) and in the hydrolysis of N-methylalanyldiglycine and sarcosyldiglycine by *B. megatherium* peptidases. The reason for this is not clear.

*The peptidases of anaerobes*

In the investigation of the 7 aerobes, the characteristics of the peptidases did not appear to differ markedly from the properties of known peptidases. The enzymes hydrolyzed their peptide substrates at pH optima close to pH 8, and were reasonably stable on incubation at this pH value and 40°. There was no particular reason to suspect that a procedure different from the one used would give better results. However, in the study of 5 anaerobes, differences, apparently in the nature of the peptidases, soon became obvious. With further investigation, the number of these differences increased until it was realized that anaerobes contained peptidases which could not be studied properly under the same conditions that were used for the aerobes. These differences in stability, pH optima and activation by reducing agents will be pointed out for the organisms where they appeared.

*Clostridium sporogenes* and *Closteridium butylicum*. 11.8 grams wet cells of *C. sporogenes* (from 14 liters of medium) were frozen 1 week, thawed 5 times and autolyzed 10 hours. 18.6 grams wet cells of *C. butylicum* grown on a 0.5 per cent tryptone, 0.5 per cent glucose medium, were frozen 3 months, then autolyzed for 79 hours. The analyses of these enzyme solutions are given in table 9 (which also includes data for *Clostridium acetobutylicum*). When cells of *C. butylicum* grown on the regular peptone-glucose medium were frozen 1 week and autolyzed 10 hours, 2.7 and 8.5 units of AGG- and LGG-hydrolyzing enzymes respectively per 10 grams wet cells were obtained. In an incubation period of 2 hours, no hydrolysis of the other 4 peptides tested was obtained.

We noticed in our work a marked instability of the peptidases of these two organisms and also of certain other anaerobes. In

a previous paper (Berger, Johnson and Peterson, 1938) it was shown that the unstable AGG-hydrolyzing enzyme of *Leuconostoc mesenteroides* was activated by a large number of heavy metals. It has subsequently been found that reducing agents such as thioglycolic acid, cysteine, $H_2S$, HCN and monomethyl-paramido phenol sulfate ("metol") also have a marked activating effect on this peptide hydrolysis. Data are given in table 10. The AGG-hydrolyzing peptidases of *C. butylicum* (and *Lactobacillus*

TABLE 9

*Peptidases of C. sporogenes, C. butylicum and C. acetobutylicum*

| SUBSTRATE | C. SPOROGENES* | | C. BUTYLICUM† | | C. ACETOBUTYLICUM‡ | | |
|---|---|---|---|---|---|---|---|
| | Hydrolysis | Yield per 10 grams wet cells | Hydrolysis | Yield per 10 grams wet cells | Time of incubation | Hydrolysis | Yield per 10 grams wet cells |
| | *per cent* | *cc.* | *per cent* | *cc.* | *hours* | *per cent* | *cc.* |
| *dl*-AG | 2 | 0 | 22 | 3.9 | 24 | 30 | 0.5 |
| *dl*-LG | 1 | 0 | 22 | 3.9 | 24 | 26 | 0.5 |
| GG | 1 | 0 | 6 | 1.1 | 24 | 6 | 0.1 |
| *dl*-AGG | 16 | 4.9 | 14 | 2.5 | 6 | 18 | 1.3 |
| *dl*-LGG | 50 | 15 | 44 | 7.8 | 6 | 14 | 1.0 |
| GGG | 2 | 0 | 10 | 1.8 | 24 | 6 | 0.1 |

* *C. sporogenes*—Substrates contained 0.003 M $MgCl_2$. 0.5 cc. crude enzyme solution (corresponding to 82 mgm. wet cells) was used per determination. Time of incubation was 2 hours, except for GGG (4 hours)

† *C. butylicum*—0.75 cc. crude enzyme solution (corresponding to 140 mgm. wet cells) was used per determination. Incubation time was 2 hours throughout.

‡ *C. acetobutylicum*—Substrates contained 0.003 M $MgCl_2$. 1.0 cc. of crude enzyme solution (corresponding to 115 mgm. wet cells) was used per determination.

Basis for calculating yields same as in table 2.

*pentosus*) were also activated by "metol." Investigations at present under way indicate that the peptidases of many anaerobic species are activated by reducing agents. Weil and Kocholaty (1937) and Maschmann (1938) have reported activation of bacterial proteinases by reducing agents, but did not mention activation of peptidases.

The pH optimum for the hydrolysis of LGG by *C. sporogenes* peptidases was found to be 7.4 as shown in figure 1. When determinations were made after a 4-hour incubation period

instead of 1 hour, the pH optimum appeared to be 6.5 to 7.2. This, however, is probably the pH stability range. These data suggest that only determinations in which relatively short periods of incubation are used can be relied upon to give true pH activity optima. In the literature review in table 1, 24 hours was the shortest incubation period used in the determination of any of the pH optima. We have found the pH optimum for the hydrolysis of AGG in the presence of $10^{-3}$ molar "metol" by *C. butylicum* peptidases to be 7.5 (1 hour incubation at 40°).

The hydrolysis of AGG and LGG by an acetone-precipitated enzyme preparation from *C. sporogenes* was not activated by

TABLE 10

*Activation of peptidases by reducing agents (L. mesenteroides)*

| MOLAR CONCENTRATION OF ACTIVATOR | PER CENT HYDROLYSIS OF AGG | | | | |
|---|---|---|---|---|---|
| | Cysteine | $H_2S$ | HCN | Thioglycolic acid | "Metol" |
| None | 27 | 27 | 27 | 27 | 34 |
| $10^{-2}$ | 104 | 66 | 66 | 73 | 92 |
| $10^{-3}$ | 29 | 44 | 54 | 106 | 94 |
| $10^{-4}$ | 29 | 79 | 42 | 30 | 96 |
| $10^{-5}$ | 29 | 88 | 36 | | 71 |

The same enzyme solution gave 80 per cent hydrolysis of AGG in the presence of $10^{-3}$ M $Zn^{++}$.

Incubation was for one hour.

0.003 M $Mg^{++}$ at pH 8. Thirty-one per cent of the LGG-splitting enzyme was recovered after the acetone precipitation.

*Clostridium acetobutylicum.* 13.8 grams wet cells from 14 liters of medium were frozen 6 weeks with intermittent thawing, then autolyzed with toluene for 24 hours. The data obtained on the analysis of the enzyme solution are contained in table 9. The low yields of enzyme are possibly due to the inefficiency of the extraction method as well as to the instability of the peptidases in the absence of suitable activating agents.

*Lactobacillus pentosus* and *Propionibacterium pentosaceum.* 26.7 grams wet cells of *Propionibacterium* and 10.9 grams wet cells of *Lactobacillus* were obtained per 18 liters of a medium which

contained in addition to the usual components, 0.5 per cent of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) and 100 cc. of yeast water[5] per liter of medium. The pH of this medium at the time of inoculation was about 5.8. Cells of both organisms were frozen 3 months with intermittent thawing, then autolyzed with toluene for 24 hours. The enzyme solutions were dialyzed 10 hours. The analyses of these solutions are given in table 11. Unfortunately, these data do not give the true picture of the distribution of the peptidases in these organisms since the pH optima for the hydrolysis of AG by *Propionibacterium* and of LGG by *Lactobacillus* were found to be 5.8 and 5.5, as shown in figure 6,

TABLE 11

*Peptidases of Lactobacillus and Propionibacterium*

| SUBSTRATE | L. PENTOSUS | P. PENTOSACEUM |
|---|---|---|
| | Hydrolysis | |
| | *per cent* | *per cent* |
| *dl*-AG | 6 | 20 |
| *dl*-LG | 10 | 14 |
| GG | 6 | 10 |
| *dl*-AGG | 6 | 6 |
| *dl*-LGG | 3 | 0 |
| GGG | 0 | 2 |

Time of incubation was 2 hours.

1.0 cc. dialyzed enzyme solution was used in each determination, corresponding to 188 mgm. wet cells of *Lactobacillus*, or 534 mgm. for *Propionibacterium*.

and not pH 8 at which value the routine determinations were made. *Propionibacterium* and *Lactobacillus* thus appear to contain acidopeptidases similar to those reported in acidoproteolytes by Gorbach (1937).

*Enzymes in the culture medium*

Considerable controversy exists concerning the secretion of proteinases and peptidases into the medium by living bacterial cells. Gorbach and Pirch (1936) have claimed that the secretion of proteinase occurs only after partial cell autolysis while Virtanen

[5] The clear extract from 200 grams pressed yeast autoclaved in 1 liter of water.

and Suolahti (1937) insist that living cells secrete proteinase. Whatever the true situation may be, we have found peptidase activity in filtrates from 24- to 48-hour cultures of proteolytic as well as non-proteolytic organisms. This is shown in table 12 from which it may be seen that filtrates of *Escherichia coli* (non-proteolytic) and *Bacillus megatherium* (proteolytic) contained very considerable amounts of peptidases. Filtrates of cultures *of Clostridium sporogenes, Clostridium butylicum, Clostridium*

TABLE 12

*Peptidases found in culture media*

| ORGANISM | PREPARATION | SUBSTRATE | TIME OF INCUBATION | HYDROLYSIS | APPROX. YIELD FROM 14 LITERS MEDIUM | |
|---|---|---|---|---|---|---|
| | | | | | Medium | Cells* |
| | | | *hours* | *per cent* | *cc.* | *cc.* |
| *B. megatherium*† | I | GG | 48 | 26 | 38 | 56 |
| | II | *dl*-LGG | 6 | 24 | 280 | 192 |
| | | *dl*-LGG | 4 | 27 | 472 | 192 |
| | III | *dl*-LG | 10 | 54 | 378 | 68 |
| | | *dl*-LGG | 5 | 52 | 728 | 192 |
| | | GGG | 10 | 8 | 56 | 6 |
| *E. coli*‡ | | GG | 24 | 26 | 23 | 170 |
| | | *dl*-LGG | 18 | 56 | 65 | 115 |

* Values for enzyme yields from cells were taken from tables 5 and 6. 25 grams wet cells of *E. coli* and 40 grams wet cells of *B. megatherium* were taken as average cell yields per 14 l. medium.

† 1 cc. filtrate of 24 hour cultures was used per determination.

‡ 3 liters of filtrate from a 45 hour culture were concentrated in vacuo (below 40°) to 900 cc.: this was dialyzed 18 hours, then 1 cc. used per determination.

Basis for calculating yields same as in table 2.

*acetobutylicum, Proteus vulgaris, Bacillus subtilis* and *Bacillus mesentericus* did not contain sufficient LG- or LGG-splitting peptidases to give increases in titration in 10- to 24-hour incubation periods that were significantly greater than experimental error. Cells of these organisms, if analyzed for in the same proportional dilution, would also have given no significant titration, so it is impossible to conclude in these cases whether cells or medium contained more enzyme.

## DISCUSSION

In a wide survey of this kind, optimum conditions for analysis of all the organisms could not be maintained. For the aerobes, the general procedure adopted proved to be very satisfactory. When the anaerobes were subjected to this procedure, however, it was found that their peptidases had properties differing very considerably from those of any previously studied peptidases. The instability under the experimental conditions employed, the activation by reducing agents and heavy metals, and the acidic pH optima of some of the peptidases greatly increased the difficulties involved in their analysis. This study has outlined the general properties of a large number of bacterial peptidases and also suggested the peculiar properties possessed by some peptidases. These characteristics must be kept in mind in a detailed investigation of any one species of bacteria.

## SUMMARY

1. A survey has been made of the peptidases in cell-free extracts of twelve bacterial species, including *Escherichia coli*, *Bacillus megatherium*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Bacillus mesentericus*, *Bacillus subtilis*, *Clostridium butylicum*, *Clostridium sporogenes*, *Clostridium acetobutylicum*, *Lactobacillus pentosus*, *Propionibacterium pentosaceum* and *Phytomonas tumefaciens*.

2. An extraction procedure has been found, involving repeated freezing and thawing of cells, followed by a toluene autolysis, which gives satisfactory enzyme preparations with a degree of activity which is measurable within short periods of time.

3. The pH optima have been determined for the hydrolysis of AG by 2 organisms, LG by 3 organisms, GG by 2, AGG by 2, LGG by 7 and GGG by 1. In most cases the optimum value for peptide hydrolysis was between pH 8 and 9. Two organisms, however, *Lactobacillus pentosus* and *Propionibacterium pentosaceum* were found to contain acidopeptidases which split their substrates at optimum pH values of 5.5 to 6.0.

4. In most cases the peptidases were quite stable at pH 8

and 40° but the LGG-splitting enzymes of the anaerobes (*Clostridium sporogenes, Clostridium butylicum*) were very unstable under these conditions. This instability was partly overcome by the addition of reducing agents, which for *Clostridium butylicum* activated the hydrolysis of AGG and LGG.

5. The specificity of the peptidase systems of *Bacillus megatherium* and *Escherichia coli* was studied in some detail. Dipeptides and tripeptides were readily hydrolyzed but acylated or decarboxylated peptides were hydrolyzed only very slowly or not at all. Substitution of a methyl group for a hydrogen atom of the free amino group on a peptide resulted in a very marked decrease in hydrolysis.

6. In four organisms, namely *Escherichia coli, Proteus vulgaris, Pseudomonas fluorescens* and *Phytomonas tumefaciens* a leucylpeptidase-like enzyme was found whose hydrolysis of LGG was activated by 0.003 M $Mg^{++}$.

7. Appreciable amounts of peptidases were found in culture filtrates of *Escherichia coli* and *Bacillus megatherium*. With *Escherichia coli*, more enzymes could be extracted from the cells than from the medium on which they were grown, as might be expected with a non-proteolytic organism. With the proteolytic organism *Bacillus megatherium*, more peptidases were consistently found in a given volume of medium than could be obtained from the cells grown on the same volume of medium.

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# A NEW METHOD FOR THE DETERMINATION OF DISINFECTION RATES

MOSES L. ISAACS

*DeLamar Institute of Public Health, Columbia University, New York City, New York*

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In the examination of disinfectants, the rate of killing of bacteria is implicitly contained in the result, even when that result is expressed in any such standard form as the phenol coefficient. This fact is not apparent and is not generally recognized. Let it be recalled, however, that the time to kill is the basic determination upon which the coefficient, or any other relative measure of efficiencies, is erected. "Time to kill" is related to "rate of travel," although in the killing reaction the relation is not quite so simple. It is defined by the value $k$ in the monomolecular reaction formula

$$k = \frac{1}{t} \log \frac{I}{S}$$

where $t$ = the time of contact between disinfectant and organism,

$I$ = the number of organisms at the beginning of the exposure,

$S$ = the number of survivors at the end of the exposure.

With many materials in common use, the rate is so rapid that the usual experimental techniques fail to yield any survivors (no numerical value of $S$) so that neither the value of $k$ nor any "end-point" can be determined. In common practice an attempt is made to overcome the difficulty by dilution of the disinfectant, since, in general, the greater the dilution the slower is the disinfectant action. In the widely-used phenol coefficient technique a predetermined rate of disinfection is chosen and the

dilution at which this rate exists is found by experiment. The difficulties introduced by the use of dilution, however, are many, involving differences between one disinfectant and another as well as variability, with a given disinfectant, in the rate of change of $k$ with concentration. An inspection of the equation of rate shows that if $k$ is large the experimenter faces the necessity of making the time of contact very small or the initial number of organisms very large. Because of the peculiar nature of the formula which involves the logarithm of the initial number of organisms but not the logarithm of the time, the choice, as will be demonstrated below, is limited to a manipulation of the time variable. With this in mind, an apparatus has been devised in which the time of contact between disinfectant and organism may be made as short as 0.11 second which is less than one one-hundreth of the period of contact obtainable with the methods in use up to the present time.

The reason for the choice of a small value for $t$ rather than the use of large initial numbers of organisms may be made clear by the substitution of numerical values in the equation of rate. If $k$ is assumed to be 300 for a one-minute technique, the initial number of organisms, in order to have 1 survivor, would have to be $1 \times 10^{300}$ organisms. This number of organisms is beyond all human comprehension. In a closely packed condition, such a mass of bacteria would fill our universe billions and billions of times. If the technique is so designed that the unit of exposure becomes 0.1 minute or 6 seconds, the initial number of organisms would have to be $1 \times 10^{30}$ organisms per unit volume. This is also beyond the range of practical conception. If the time of exposure is again reduced, let us say to 0.6 second or to one-tenth of the last value, the initial number required for a survival of 1 organism becomes $1 \times 10^{3}$ power or 1,000 organisms, a number which is easily obtainable and which in addition, may be increased considerably, permitting the survival of a larger number of organisms and a consequent increase in the reliability of the results. With the apparatus to be described, the short contacts between organisms and disinfectant allow the use of small volumes of materials and the determination of values of

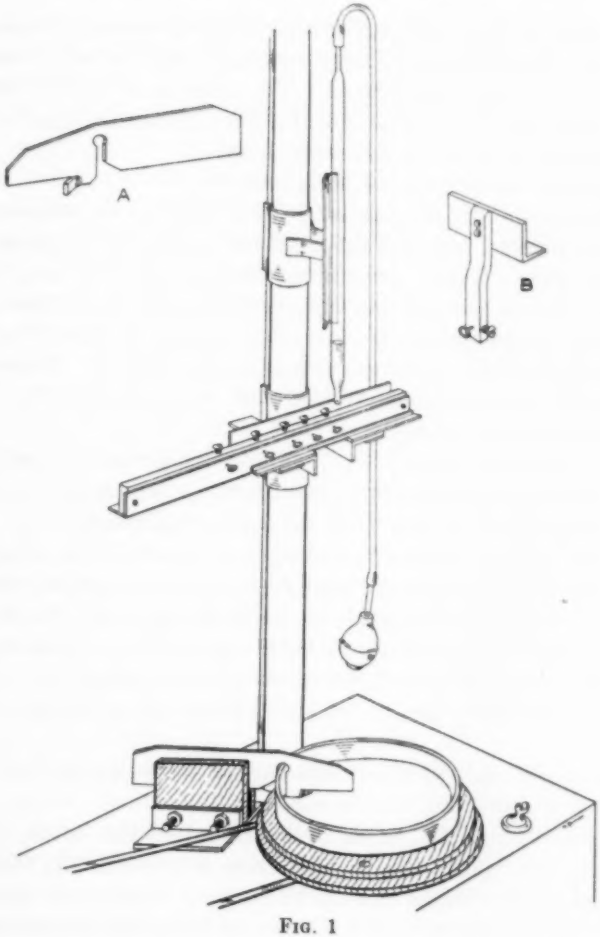


FIG. 1

$k$, even in excess of the figure used in the calculations made above.

## APPARATUS

The apparatus[1] consists essentially of a pipette from which a drop of disinfectant may be released, a platinum loop placed

[1] The apparatus may be obtained from J. H. Emerson, Cambridge, Mass.

in the path of the falling drop and below these, on a revolving turntable a petri dish containing water or a solution which arrests the action of the disinfectant. The dish is provided with a baffle which aids in the mixing of the drop, containing disinfectant and culture, with the neutralizing agent.

Considered in more detail the parts are:

1. *The pipette for disinfectant.* This may be an ordinary 10 cc. Mohr pipette, but the tip must be broken off or ground so that the drop emerges from an opening of about 2 mm. in diameter. The drop is released by gentle pressure on a rubber bulb connected to the pipette by means of a piece of rubber tubing. The volume of the drop is approximately 0.07 cc. When the disinfectant is subsequently mixed with the culture in the loop, it is diluted to the extent of 1:1.07.

2. *The platinum loops.* In the apparatus pictured, 5 of these are mounted in a removable loop carrier. The loops have an inside diameter of 2.5 mm. and are made of 22 gauge wire. The loop carrier has a series of notches (not shown in the diagram) which engage a spring on its holder, thus permitting the accurate centering of each loop beneath the tip of the pipette. The holder of the loop carrier is adjustable both vertically and horizontally. It is adjusted in the usual set-up so that the loops are 15 cm. above the surface of the liquid and 2.5 cm. below the tip of the pipette.

3. *The petri dish, turntable and baffle.* Pyrex dishes (15 mm. depth) have been found to be most uniform.

The structure of the baffle is illustrated in the figure. It is placed at an angle of $22\frac{1}{2}$ degrees from a line running through the center of the upright and the turntable. It serves to counteract the effect of the centrifugal force on the water and facilitate the mixing.

The turntable is so placed that the falling drop hits the surface of the liquid about 2 to 3 cm. from the side of the dish. It revolves clockwise at a speed of approximately 100 r.p.m. At a speed much below this figure, the mixing is not satisfactory, while at higher speeds other difficulties may be encountered.

4. *Pipette for organisms.* In addition to the apparatus shown in the figure there is needed a 0.1 cc. pipette graduated in hun-

dreths of a cubic centimeter. The graduations should be about 1.5 cm. apart. This pipette is used to charge loops.

### PROCEDURE IN MAKING A TEST

In carrying out a test the procedure is as follows. A sterile petri dish containing 15 cc. of water or other neutralizing agent (thiosulphate if iodine is being tested, etc.) is put on the turntable and the baffle, which has previously been sterilized by flaming, is put into place. Disinfectant is put in the pipette. The loops are next flamed and each charged with 0.005 cc. of suspension of organisms. This is done by allowing 0.005 cc. of suspension to form a drop on the tip of the 0.1 cc. pipette. The drop is transferred to the loop by contact. The first loop is brought into position under the pipette and a drop of disinfectant is released from the latter by a gentle pressure on the rubber bulb. The next loop is then placed in position and a second drop of disinfectant allowed to fall, this process being repeated as many times as desired. The limit in any case is a concentration of disinfectant strong enough to act as an inhibiting agent later on, or one which may continue the disinfectant action in the dish. The contents of the dish are plated out in agar in suitable quantities or dilutions so that on incubation a count may be made of the number of organisms surviving the action of the disinfectant under test.

The initial number of organisms may be determined best by the use of the technique just described except that water or broth is placed in the pipette instead of the disinfectant.

After the plates are incubated and counted the results may be expressed in terms of a percentage survival and comparisons between different disinfectants may be made on this basis. Since the apparatus as described makes no provision for temperature control, it is necessary to work in a space in which the temperature is reasonably constant.

### DETERMINATION OF $k$ VALUES

While the procedure just described enables one to determine a percentage survival under a specified set of conditions, one more value is needed,—the time of contact,—if the equation

given in the first paragraph is to be solved for $k$. The well-known formula for falling bodies, $s = \frac{1}{2} gt^2$, gives the time required for the drop to fall from the pipette to the dish as well as that required for the fall from the pipette to the loop, the difference being the time for the fall from the loop to the dish. This calculated value however, is not the value of $t$ in the equation, for it must be remembered that the action of the disinfectant continues while it is undergoing dilution. At the instant the drop hits the liquid in the dish the disinfectant action is at its maximum but as mixing and dilution proceed the action falls until it becomes so slow that it may be neglected. The value of $t$ needed for the equation must sum up the whole disinfectant action in terms of undiluted disinfectant. This may be called the "equivalent" time of mixing.

The determination of this hypothetical time is fortunately not difficult, the method of calculation being seen most easily in a description of an actual experiment and the use of the data obtained from it. First, a determination of the survival after a 15 cm. fall, according to the method already described, was made, with 5 per cent phenol and *Staphylococcus aureus*. Next an experiment was run in which the special loop holder (*B* in the figure) was used. The loop was placed about 8 mm. from the surface of the liquid, and the tip of the pipette about 8 cm. above the loop. The purpose of this arrangement was to make the time of contact between organism and disinfectant in the air a negligible quantity, so that any organisms killed might safely be assumed to have been acted upon during the process of dilution of the disinfectant in the dish. The data obtained were as follows:

| | |
|---|---|
| Initial number of organisms | 3,200 |
| Survivors after 15 cm. drop | 680 |
| Survivors after immediate mixing | 1,600 |

It will be seen that the process of mixing alone leaves 50.0 per cent of the organisms surviving. In other words it may be inferred that the 680 organisms surviving from the 15 cm. fall represent only 50.0 per cent of the organisms which were living at the instant when the drop of disinfectant and culture hit the

surface of the water in the dish. This number was therefore $2 \times 680$ or 1360 organisms. Since the calculated time for falling 15 cm. (after the drop has already fallen 2.0 cm.) is 0.122 second,[2] we may proceed to calculate the $k$ of the reaction. Substituting in the formula we have

$$k = \frac{1}{0.122} \log \frac{3200}{1360}$$

$$k_{(\text{sec.})} = 3.04$$

Now, having the value of $k$, we may calculate $t$, the equivalent time of mixing, by substituting the proper values in the same basic equation, which gives,

$$3.04 = \frac{1}{t} \log \frac{3200}{1600}$$

$$t = 0.099 \text{ sec.}$$

The actual time of mixing therefore is equivalent to 0.099 second exposure to full-strength disinfectant. A series of eight experi-

[2] For convenience in estimating the time of fall the following solutions of the equation $s = \frac{1}{2}gt^2$ are given.

| s | TIME (t) | s | TIME (t) |
|---|---|---|---|
| cm. | second | cm. | second |
| 0.5 | 0.032 | 15 | 0.174 |
| 1.0 | 0.045 | 16 | 0.181 |
| 1.5 | 0.055 | 17 | 0.186 |
| 2.0 | 0.064 | 20 | 0.202 |
| 2.5 | 0.071 | 25 | 0.226 |
| 3.0 | 0.078 | 30 | 0.247 |
| 4.0 | 0.090 | 31 | 0.251 |
| 5.0 | 0.101 | 32 | 0.255 |
| 10.0 | 0.142 | | |

As an illustration of the use of the table let us consider the following set-up. The tip of the pipette is placed 2.5 cm. from the loop. The loop is 15 cm. above the liquid in the receiving dish, the diameter of the drop emerging from the pipette is 0.5 cm., the drop therefore falls 2 cm. to the loop and 15 cm. to the dish making a total of 17 cm. fall. Referring to the table, the time required to fall 17 cm. is 0.186 second. The time required to fall the 2 cm. is 0.064 second. During the last mentioned period of time, the drop of culture is falling unmixed with disinfectant and therefore must be subtracted from the total giving us 0.122 second. When this figure is added to the mixing time of 0.11 second, the total time of contact, in this case 0.23 second, is obtained.

ments gave the following values of $t$, 0.088, 0.099, 0.100, 0.107, 0.113, 0.114, 0.114, 0.140, the average value being close to 0.11 sec. If then, the apparatus is set up as described with a 15 cm. fall (the drop first falling 2.0 cm. measured from the bottom of the drop) the total contact of disinfectant and organism is 0.122 + 0.110 second or approximately 0.23 second. The time of mixing may be expected to vary somewhat with the rate at which the disinfectant is inactivated by dilution, the concentration of the disinfectant and nature of the neutralizing medium in the receiving dish.

### TESTS OF ACCURACY AND REPRODUCIBILITY OF RESULTS

In calculating the time of contact from the formula $s = \frac{1}{2} gt^2$ the assumption was made that mixture takes place completely and instantaneously when the drop hits the culture contained in the loop. This need not necessarily be true, for it is conceivable that the drop of disinfectant might push the culture ahead of it and mixture might occur only after some distance of fall. It was therefore necessary to test this point experimentally and for this purpose a chemical test was devised. Water was placed in the pipette and 0.005 cc. of normal hydrochloric acid was placed in the loop. Ten drops of water were allowed to fall, as in the bacteriological technique, on ten loops of acid and the acid remaining on the loops and that falling into the dish was titrated. Titration was carried out with N/100 sodium hydroxide. Theoretically, if perfect mixing had occurred during the passage of the water drop through the loop, the concentration of acid in the drop left in the loop should have been the same as the concentration of the acid in the drop which fell into the dish. In a series of nine determinations the average concentration of acid for the drop in the loop was 0.077 N, while that of the drop which continued toward the dish was 0.067 N. These figures indicate that a satisfactory mixing of disinfectant and culture occurs during the extremely short time required for the disinfectant to pass through the loop and therefore the time of contact may be assumed to begin at the instant the drop leaves the loop.

A number of factors are involved in the reproducibility of results. The most important of these are, the constancy of

resistance of the organism used, the homogeneity of the suspension (absence of clumps) and the error inherent in the plate count. With one suspension of a filtered culture of *Staphylococcus aureus* diluted in extract broth, the results given in table 1 were obtained. The number of survivors (average of two duplicate plates for each experiment) varied from 57 to 70 with an over-all average of 64. All of these variations are well within the error of the plate count. The variation in cultures made from day to day for the same organism in the same medium is much more marked than that given in the table above. For the organism used in the experiments outlined above the average $k$ values for individual broth cultures have run as low as 2.5 and as high as 4.

TABLE 1

*Action of 5 per cent phenol on Staphylococcus aureus*

| INITIAL NUMBER | NUMBER OF SURVIVORS (AVERAGE OF 2 PLATES) | PER CENT SURVIVAL | $k$ |
|---|---|---|---|
| | | | seconds |
| 260 | 57 | 21.9 | 2.87 |
| | 64 | 24.6 | 2.65 |
| | 64 | 24.6 | 2.65 |
| | 66 | 25.4 | 2.59 |
| | 70 | 26.9 | 2.48 |

## APPLICABILITY OF THE MONOMOLECULAR FORMULA

In devising the apparatus, the applicability of the monomolecular reaction formula was assumed. There are a number of instances reported in the literature, however, where different orders of reaction have been found to prevail and in some cases the reaction rate appears to follow some special formula which probably includes, besides the number of organisms and concentration of disinfectant, a penetration factor. Furthermore, a number of workers have been of the belief that the formula has only an apparent application and that, if shorter times of contact were possible, there would be demonstrated a short period during which the reaction follows a different path.

In order to test whether a reaction is of the first order, that is, of the monomolecular type, two criteria are available. The

first involves the constancy of $k$ with variation in the initial number of molecules or organisms, the second the constancy of $k$ with variation in time of exposure. Experiments were performed to test each of these criteria and the results obtained, while open individually to certain objections, pointed out below, indicate that a reaction of the first order takes place in the time of contact used in the apparatus.

In order to test the first criterion, a series of dilutions of culture was made in broth and each tested out against 5 per cent phenol in the apparatus. The results of a typical experiment are given in table 2. The variations in the per cent survival (20.8 to 25.4), and the corresponding $k$'s (which varied from 2.58 to 3.12) are close enough together to warrant the conclusion that the rate is independent of the initial number of organisms. From this

TABLE 2

*Results of experiments with different initial numbers of organisms*

| INITIAL NUMBER OF ORGANISMS | FINAL NUMBER | PER CENT SURVIVAL | $k$ |
|---|---|---|---|
| | | | *seconds* |
| 260,000 | 66,000 | 25.4 | 2.58 |
| 26,000 | 5,000 | 19.2 | 3.12 |
| 2,600 | 540 | 20.8 | 2.96 |
| 260 | 60 | 23.1 | 2.76 |

result the further conclusion may be drawn that the reaction of phenol and staphylococcus is one of the first order, provided of course, that the premise is granted that a reaction is occurring which has a rate which is either mono-, bi- or tri-molecular.

The second criterion, which involves experimentally the variation of time of contact, while representing a better test, is difficult to apply since the limitations of the apparatus do not permit a very great range of times of exposure. By permitting a drop to fall 1 cm. before hitting the loop and the mixture of disinfectant and organisms to fall 30 cm. before being neutralized in the dish, a time of contact in the air of 0.21 second was obtained which, together with the equivalent mixing time of 0.11 second, yielded a total exposure of 0.32 second. In a second experiment the drop was permitted to fall 2 cm. and a further 15 cm. after hitting the

loop. The times of contact were 0.12 in the air and 0.11 in the dish making a total of 0.23 second. With 4100 organisms as the initial number, the survivors were respectively 205 and 500 organisms, the $k$ values being approximately 3.9 in each case. The difference in time of contact, while apparently not very large, was yet sufficiently great to yield very definite differences in the number of survivors. The conclusion may be drawn that the reaction between phenol and staphylococcus proceeds at a monomolecular rate over a period of time. The results also strengthen the correctness of the assumptions made in the calculation of the equivalent mixing time.

In the interpretation of the results as a whole, a word of caution may be in place. While it has been demonstrated above that in the general neighborhood of 0.2 second contact, the reaction between phenol and staphylococcus proceeds as a monomolecular reaction, there may still be a very short period of contact, perhaps in the nature of 0.01 of a second, during which some other, or perhaps even no action at all, takes place. Secondly, in our use of 1 second as the unit of time there is implied the assumption that the reaction proceeds with unvarying rate for a period at least 5 times longer than that under which the actual experiments have been carried out. Such an assumption need not necessarily be correct since it is conceivable that the reaction rate might change suddenly after a given period of time. Observations of this sort are common in survival curves run with the usual techniques. Finally, the relationships demonstrated between the staphylococcus and phenol may not be carried over without experimental proof to other organisms and other disinfectants. That the reaction between staphylococcus and phenol, however, is not unique has been shown by results obtained with iodine and *Escherichia coli* to be reported later.

## SUMMARY

An apparatus for the study of the disinfection process is described, in which contact between organism and disinfectant may be made for periods of about 0.2 second. The set-up permits the determination of disinfection rates of strong disinfectants without appreciable dilution.

# HETEROAUXIN AND THE GROWTH OF ESCHERICHIA COLI

ERNEST BALL

*Department of Botany, University of Oklahoma, Norman, Oklahoma*

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The possibility that heteroauxin (3-indoleacetic acid) which is produced by bacteria may have a stimulating effect upon the growth of the organism producing it apparently has not been investigated. *Escherichia coli* was chosen for an investigation of this problem since it has been found to produce heteroauxin under appropriate conditions (Hopkins and Cole, 1903).

E. Salkowski and H. Salkowski (1880) and E. Salkowski (1885, 1885a), working with mixed cultures in the putrefaction of proteins, found that 3-indoleacetic acid was associated with the growth of bacteria. *E. coli* produces 3-indoleacetic acid in addition to indol by the breakdown of tryptophane (Hopkins and Cole, 1903). Herter (1908) demonstrated that 3-indoleacetic acid found in the urine of patients came from bacteria that were active in the putrefaction of intestinal contents. This acid was absorbed through the intestinal wall and excreted by the kidneys. Herter and Ten Broek (1909) found that *Proteus vulgaris* produced 3-indoleacetic acid and indol from a peptone-beef extract medium.

Frieber (1921) reported that heteroauxin is produced during the fermentation of tryptophane by both indol-positive and indol-negative bacteria. The breakdown of tryptophane to indol occurs in two stages. During the first stage, 3-indoleacetic acid is produced and in the second stage this acid is converted into indol. Indol-positive bacteria, in their fermentation of tryptophane in the absence of sugar as a source of carbon, carry the fermentation through both stages to produce first 3-indoleacetic acid and then indol. When indol-positive bacteria are

grown in artificial media with tryptophane, and sugar as the preferred source of carbon, only the first stage in the breakdown of tryptophane is carried out and 3-indoleacetic acid is the final product. Indol is produced from the fermentation of tryptophane by indol-positive bacteria only when the sugar supply is exhausted. 3-indoleacetic acid is but a stage in the process of indol formation; it cannot serve as proindol. Indol-negative bacteria form 3-indoleacetic acid from tryptophane, but the process never, of course, culminates in the production of indol. The second stage of the process, the conversion of 3-indoleacetic acid to indol, does not occur. Sugar merely stimulates the growth of indol-negative bacteria by providing additional nutrient and does not effect the manner in which tryptophane is broken down; in the presence of sugar as the carbon source the process still goes through only the first stage to produce 3-indoleacetic acid.

Nielsen (1930b) has shown that rhizopin[1] accelerates the growth velocity of yeast. Boysen Jensen (1931) found that bacteria from human saliva, *E. coli*, and other bacteria produced growth substances which, when applied in an agar block to one side of an oat coleoptile, caused it to bend negatively. An abundant formation of growth substance was brought about only with growth of bacteria upon solid media. It is probable that this growth substance, like rhizopin, is heteroauxin (Went and Thimann, 1937).

Boysen Jensen (1931a)[2] mentions the possibility that the "growth regulator" produced by microorganisms may accelerate their growth velocity. If this is true the presence of such a substance in media would be advantageous since growth of bacteria in such media occurs at a faster rate. However, Went and Thimann (1937) point out that, so far as is known, the 3-indoleacetic acid produced by fungi has no effect upon their growth. No mention is made of the possibility that this hormone may have an influence upon the growth rate of bacteria.

[1] Thimann (1935a) has demonstrated rhizopin to be 3-indoleacetic acid.
[2] Footnote, page 245.

## MATERIALS AND METHODS

Cultures of *Escherichia coli* were grown in a synthetic liquid medium in order to avoid the effects of organic growth factors other than heteroauxin. The constituents of this medium[3] were:

| | |
|---|---|
| $K_2HPO_4$ | 0.50 gram |
| $KH_2PO_4$ | 0.50 gram |
| $MgSO_4 \cdot 7H_2O$ | 0.20 gram |
| NaCl | 0.01 gram |
| $FeSO_4 \cdot 7H_2O$ | 0.01 gram |
| l-tryptophane[4] | 0.10 gram |
| Glucose | 10.0 grams |
| Distilled water | 1 liter |

3-indoleacetic acid[4] was added to this medium in concentrations ranging from one to ten parts per ten million parts of medium. The pH of the culture fluid was 6.4.

Inoculations were made from 24-hour-old cultures of *E. coli*, that had been grown in nutrient broth at 37°C., into tubes that contained 5 cc. of the synthetic liquid medium. These source cultures contained approximately 2,700,000 cells per $mm^3$. The volume of the loop used in inoculations was approximately 0.8 $mm^3$ and contained approximately 2,160,000 bacterial cells.

Counts were made of 48-hour-old cultures in the synthetic medium that had been incubated at 37°C. The apparatus used was a Petroff-Hauser bacterial counting chamber with an oil immersion microscope. Cultures were grown and counted in triplicate for each concentration of heteroauxin from one to ten parts per ten million of culture fluid. For controls, counts were made of cultures grown under the same conditions and in the same medium containing no heteroauxin.

## EXPERIMENTAL RESULTS

The experimental results are given in table 1.

## DISCUSSION AND CONCLUSION

The counts of *E. coli* given show that concentrations of heteroauxin between one and ten parts per ten million parts of culture

[3] Adapted from Snell, Tatum and Peterson, Jour. Bact. **33**, 207, 1937.
[4] Obtained from Eastman Kodak Company.

fluid give approximately the same quantitative stimulus to cell division. The number of cells was more than doubled in the medium containing the hormone as compared with the number of cells in control tubes that contained no hormone. These figures indicate that growth, as measured by cell division, is not proportional to the concentration of heteroauxin within these limits. Therefore, the major function of heteroauxin is apparently that of a stimulus to cell division in *E. coli*. In the experiments outlined, the bacterial cells divided faster in the presence of artificially added heteroauxin than they did without it.

TABLE 1

*Experimental results*

| CONC. P.P. $10^7$ OF HETEROAUXIN | COUNT 1, CELLS PER $MM^3$. | COUNT 2, CELLS PER $MM^3$. | COUNT 3, CELLS PER $MM^3$. | AVERAGE |
|---|---|---|---|---|
| 0 | | | | 92,000* |
| 1 | 190,000 | 226,000 | 228,000 | 214,000 |
| 2 | 222,000 | 226,000 | 202,000 | 216,000 |
| 3 | 190,000 | 226,000 | 214,000 | 210,000 |
| 4 | 200,000 | 226,000 | 266,000 | 230,000 |
| 5 | 182,000 | 208,000 | 266,000 | 218,000 |
| 6 | 186,000 | 216,000 | 238,000 | 214,000 |
| 7 | 156,000 | 222,000 | 240,000 | 206,000 |
| 8 | 236,000 | 194,000 | 218,000 | 216,000 |
| 9 | 204,000 | 202,000 | 228,000 | 212,000 |
| 10 | 220,000 | 202,000 | 212,000 | 212,000 |

* Average of 17 counts.

As to the effect of heteroauxin upon the size of the bacterial cell and upon the morphology of cells in old cultures, no studies were made. It is quite possible, however, that heteroauxin may influence the size of the bacterial cell and also that it may have a rôle in the induction of pleomorphism. It is left for future studies to determine this.

Hopkins and Cole (1903) have shown that *E. coli* produces 3-indoleacetic acid from tryptophane. The results of this investigation show that there is a pronounced stimulus brought about in the growth of the organism by the hormone. The relation of heteroauxin to the growth of this organism appears therefore to be that of a self-activating system; heteroauxin is produced

in the normal growth processes of these bacterial cells by their oxidative deamination of tryptophane (Went and Thimann, 1937). The heteroauxin so formed apparently stimulates the cells to further growth and division which produces more cells and more heteroauxin.

Boysen Jensen (1932) established that *Aspergillus niger* was able to produce growth substances by the breakdown of the following amino acids besides l-tryptophane: l-histidine, l-leucine, d,l-phenylalanine, d-lysine and l-tyrosine. When agar blocks containing these growth substances were applied to one side of an oat coleoptile, negative curvatures resulted. The possibility must be considered that bacteria, in fermenting amino acids other than tryptophane, also produce growth substances that should cause bending of the oat coleoptile as well as an acceleration of their own growth velocity.

This mechanism of growth stimulus for a bacterium may be compared to that which was postulated by Söding (1937) for the effect of auxin of higher plants upon cambial growth. He presented evidence that the growth substance, which comes from the bud, is conducted downward for a short distance in the stem only in the cambium. In the cambial cells exposed to the auxin, there is a stimulation of growth and cell division which produces more auxin. The auxin so formed is again conducted downward a short distance in the cambium where the cycle of initiation of growth and the production of additional auxin comes about: the wave of growth that goes down the stem is thus explained.

The stimulation of the bacterial cell by heteroauxin to produce more heteroauxin and normal growth by cell division may also be compared to one theory of virus action. As postulated by Schultz (1930) and Rivers (1932), a virus-tainted cell is stimulated to the production of more of the same agent which induced the original disease process and the abnormal growth of the cells characteristic of the disease.

## SUMMARY

*Escherichia coli* was grown in a synthetic culture fluid which contained from one to ten parts of heteroauxin per ten million

parts of fluid. Counts were made in triplicate of cultures of each of these concentrations of the hormone. Regardless of the concentration of the hormone used in the medium the same stimulus of cell division was obtained. The cell division of the organism was more than doubled in the medium containing the hormone as compared with that in control tubes containing no hormone.

Comparisons are drawn with Söding's theory of the action of growth substances in the cambium of higher plants and with a modern theory of the action of viruses.

I wish to make acknowledgments for favors done in the course of this work to Dr. H. L. Chance, under whose direction the work was carried out, to Mrs. Leena J. MacArthur for the loan of equipment from the School of Pharmacy, to Dr. K. V. Thimann of Harvard University for valuable advice and criticism and to Dr. G. L. Cross for suggestions concerning the manuscript.

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# PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

## MISSOURI VALLEY BRANCH

OMAHA, NEB., MAY 14, 1938

THE EFFECT OF SULFANILAMIDE ON THE STREPTOCOCCI IN THE UDDER OF MASTITIS COWS. *Henry Bauer and M. F. Gunderson*, Department of Pathology and Bacteriology, College of Medicine, University of Nebraska, Omaha, Nebraska.

Eight cows infected with chronic mastitis were treated with doses of sulfanilamide ranging from one-half to sixteen ounces. These doses were fed over a period of twelve hours to twenty-two days. The amount of drug excreted was determined at hourly or daily intervals. Fuller's method for the estimation of sulfanilamide in blood was modified for the estimation of the drug in milk. Curves revealing the sulfanilamide concentration in blood and milk were obtained. The streptococcal flora of the treated cows was followed for several months by means of incubated milk samples of which Breed smears were made, blood agar plates, brom-thymol-blue and Hotis tests.

Sulfanilamide exerted a transient effect on the streptococci in the udder of the infected cows. As long as the drug could be detected in the milk, the streptococcal count remained low and in some instances disappeared; however, as soon as the udder was cleared of the drug, the streptococci reappeared in numbers as great as before treatment was begun.

One of the eight cows was infected with staphylococci; the drug showed no effect on these organisms.

MESENCHYMAL REACTIONS AND BODY DEFENSE. *Wilhelm Voigt*, University of Nebraska, College of Medicine, Omaha.

The work of Wilhelm Ehrich (Ehrich and Wohlrab, Ehrich and Voigt in Beitr. pathol. Anat., Vol. **92**, 2) has been extended. There is a relationship of the condition of immunity and the occurrence of cellular reactions of the vascular connective-tissue-apparatus. Intensive reactions of the intima and adventitia of the small vessels of lung, liver and spleen were noted upon the intravenous injection of a heat-killed culture of *Staphylococcus aureus*. The sizes of the germinal centers in lymphnodes vary directly with the agglutinin titers.

FACTORS FACILITATING THE CULTIVATION OF NON-SPORULATING ANAEROBIC BACILLI FROM THE INTESTINAL TRACT. *Margaret Bedell and Keith H. Lewis*, Department of Bacteriology, University of Nebraska, Lincoln, Nebraska.

A study of the growth-promoting properties of a variety of gases, mineral salts, carbohydrates, proteins, infusions, and extracts has led to the preparation of a relatively simple,

clear, heat-stable medium of the following composition:

| | per cent |
|---|---|
| Tryptone | 2.0 |
| Bacto beef extract | 1.0 |
| Glucose | 0.5 |
| Cysteine hydrochloride | 0.05 |
| Di-sodium phosphate (hydrate) | 0.4 |

Dissolve all ingredients in distilled water, adjust to pH 7.4, and sterilize in the autoclave for 20 minutes at 120°C.

While this medium supplies all nutrients essential for rapid development of the non-sporulating anaerobic bacilli, it has been noted also, (1) that the exclusion of carbon dioxide from the atmosphere completely inhibits growth, and (2) that the addition of liver infusion, yeast extract, and tomato juice stimulate more profuse growth.

STUDIES ON MEMBERS OF THE ESCHERICHIA-AEROBACTER GROUP ISOLATED FROM KANSAS ICE CREAM. *F. E. Nelson, B. F. Diamond, and W. J. Caulfield,* Kansas State College, Manhattan, Kansas.

Presumptive tests for presence of *Escherichia-Aerobacter* group were made by adding measured amounts of melted ice-cream to lactose peptone bile 2 per cent broth. Of the 51 samples examined, 40 yielded positive presumptive tests, 37 of which were confirmable. On each of the 24 samples in the second series, 16 of which gave confirmable tests for organisms of the *Escherichia-Aerobacter* group, phosphatase tests for pasteurization efficiency were made. Four samples were positive to both tests indicating that possibly the organisms were present because of inefficient pasteurization. Twelve samples were phosphatase negative but still contained *Escherichia-Aerobacter* organisms indicating contamination after pasteurization or the presence of resistant strains. Each colony from positive presumptive tests was isolated and studied. Of 86 cultures obtained, 54 were *Aerobacter*, 26 were *Escherichia*, and 6 were *Citrobacter*. All were killed by 30 minutes exposure at 142°F. using sterile skim milk as a suspension medium but 12 *Escherichia* survived the exposure when sterile ice-cream was used.

Indications are that organisms of the *Escherichia-Aerobacter* group are frequently present because of contamination following pasteurization, although inefficient pasteurization may be the reason for their presence under some circumstances. The use of higher temperatures than are used for the pasteurization of milk for the processing of ice cream mix is again indicated by the resistance of a number of *Escherichia*-strains to exposure to 143.5°F. for 30 minutes when suspended in ice cream mix.

SULFANILAMIDE LEVELS IN THE BLOOD OF RABBITS WITH AND WITHOUT FEVER TREATMENT. *Murray D. Lewis, M.D., and Millard F. Gunderson, Ph.D.,* Department of Bacteriology, University of Nebraska, College of Medicine, Omaha.

The recent use in man of combined sulfanilamide and fever therapy, particularly for gonorrhea, suggested the necessity for observing animals subjected to this combined treatment.

Fifteen rabbits were fed 1 gram sulfanilamide per kilo and placed in a cabinet modeled after the Kettering Hypertherm. The temperature of these animals was kept at 107–108°F. for 9–10 hours. Ten survived the treatment. The blood sulfanilamide reached higher levels than in the five that died, suggesting that the cause of

death was not due to sulfanilamide over-dosage but rather hyperpyrexia. The maximum blood sugar level was 36 mgm. per cent and this was not reached until the sixth hour contrasting with the 57 mgm. per cent at the third hour in the unheated controls. The slower rise in blood sulfanilamide in these 10 animals to a lower maximum may possibly be attributed to less rapid absorption from the gastro-intestinal tract in the presence of high fever. The fact that 10 out of 15 animals treated in this fashion survive without permanent ill-effect 9 or 10 hours of fever at 107–108°F. suggests that the feeding of large doses of sulfanilamide increases the hazard of artificial fever therapy not at all, or only slightly.

As controls, rabbits were fed 1 gram per kilo of sulfanilamide but were not put in the fever cabinet. These animals all showed signs of shock with cyanosis, coldness, and fall in rectal temperature from 101–102°F. to 99–100°F. The figures for blood sulfanilamide levels in these animals is given above.

THE INFLUENCE OF ADSORBED MAGNESIUM ON LEGUME BACTERIA. *T. M. McCalla*, Kansas State College, Manhattan.

A neutral colloidal clay system containing Mg, $PO_4$, K, and Ca proved superior to a similar system in which Ba was substituted for Mg as a medium for maintaining normal strains of these organisms.

DETOXIFICATION OF TYPE C BOTULINIUM TOXIN BY PROTEINS. *M. F. Gunderson*, Department of Pathology and Bacteriology, University of Nebraska, College of Medicine, Omaha, Nebraska.

Doses of three cubic centimeters of Type C botulinum toxin, 0.001 cc. of which was fatal by injection, were fed to pigeons. Death ensued in less than twelve to twenty-four hours.

Similar amounts of toxin, mixed with 0.5 cc. fresh egg-albumen, and fed to pigeons failed to kill until seven to fifteen days elapsed, and, in instances, death did not result.

The binding can be dissociated so that the toxin will quickly exert its effect in spite of the presence of protein.

FIBRINOLYTIC ACTIVITY OF STREPTOCOCCI OF HUMAN AND ANIMAL ORIGIN. *John D. Lemar and Millard F. Gunderson*, Department of Pathology and Bacteriology, University of Nebraska College of Medicine, Omaha, Nebraska.

Forty streptococci of human origin and seventy streptococci of animal origin were tested for their fibrinolytic activity upon fibrin obtained from a number of sources. These included human, horse, bovine, swine, sheep, hog, dog, guinea pig, rabbit, pigeon and duck fibrins.

The human streptococci were obtained, with a few exceptions, from superficial infections, as were those of animal origin. The veterinary sources included horse, cow, swine, pig, sheep, guinea pig, rabbit, fox, and chicken.

The streptococci of human origin were active against all fibrins, though this was not marked in the case of fibrins obtained from swine, sheep, dog, horse, and guinea pig. The greatest activity was against human fibrin.

Some activity was displayed by the animal streptococci against all fibrins. This was most marked against human fibrin. Horse and pigeon fibrin were only slightly susceptible.

In both groups of organisms, prolonged incubation (96 hours) enhanced

the fibrinolytic powers of most of the organisms. In the few exceptions fibrinolytic power was not changed, decreased, or lost entirely.

In this study there seemed to be no correlation between species-pathogenicity and specific fibrinolytic power. Fibrin of human origin was more susceptible to lysis by both groups of organisms than was any other.

CONSTITUTIONAL CHANGES IN RABBITS SUBJECTED TO COMBINED FEVER AND SULFANILAMIDE THERAPY. *Lucile Loseke and M. F. Gunderson*, University of Nebraska, College of Medicine, Omaha, Nebraska.

Normal healthy rabbits were kept for 5–6 hours in a heated cabinet with controlled humidity, their temperatures reaching 108°F. Blood taken immediately after the animals were removed from the fever cabinet showed a moderate increase in hemoglobin and red count, slight decrease in white count, and relative increase in pseudo-eosinophiles and decrease in lymphocytes. Non-protein-nitrogen was increased one-third, blood sugar levels, doubled, and $CO_2$ combining power decreased. Rabbits treated with sulphanilamide and also untreated rabbits were given fever treatments during which time red, white, differential, and blood sugar values were obtained, first at 15 minute intervals; later at hourly periods. In all cases an initial drop occurred in red and white counts during first part of fever period. The red count gradually increased after the initial drop so that it usually exceeded the normal count at the end of 75 hours of fever. The white count increased after an initial drop and the granulocytes increased at the expense of the lymphocytes. Blood sugar commenced to be increased almost at once after the exposure in the heated cabinet.

Studies were also made on rabbits treated with small daily doses of sulfanilamide for several weeks and others treated with sulfanilamide daily and fever weekly. These animals all maintained their weight as long as they lived. Blood sugar and non-protein-nitrogen showed no significant change from week to week. The carbon dioxide combining power decreased slightly. The difference between total hemoglobin and hemoglobin as determined by oxygen capacity method varied from 0.1 to 3.0 grams per 100 cc. of blood.